THE STRUCTURE AND FUNCTION OF CHOLERA TOXIN

TAMARA SUSAN SCOBIE

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PREFACE

The work reported in this thesis was carried out between 1st October 1983 and 30th September 1986 under the supervision of Dr. Simon van Heyningen at the Department of Biochemistry, University of Edinburgh Medical School, or under the supervision of Dr. Chris J. Coulsen and Dr. R. Murray Tait at the Department of Microbiological Biochemistry, Glaxo Group Research Ltd., Greenford, Middlesex. Unless stated otherwise, all material presented is the sole work of the author, as is the composition.

TO MY PARENTS

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ABSTRACT

Cholera toxin catalyses the ADP-ribosylation of N_s, a regulatory component of adenylate cyclase causing the cyclase to be permanently activated. To study this reaction a model system was used in which low molecular weight arginine derivatives were substituted for the acceptor protein. Michaelis constants calculated using this method were 4mM for NAD^+ in the absence of the arginine derivatives but 45 - 51mM in their presence while the values for the arginine derivatives themselves were 0.40 - 0.41mM. A number of inhibitors of the enzyme competitive with NAD^+ were examined, the best of these being thio-NAD^+ and acetyl pyridine adenine dinucleotide. Other alternative ADP-ribose acceptors including transducin and p-nitrobenzylidine aminoguanide were studied but found to be poor substitutes for N_s under the conditions used.

The binding of NAD^+ and the arginine model substrates to the cholera toxin's enzymatically active A_1 chain was studied using equilibrium dialysis and indirect methods based on changes in the absorbance or fluorescence of toxin or fluorescently-labelled toxin upon binding but results were inconclusive. It was noted that saturating concentrations of NAD^+ could protect the A_1 chain from digestion with trypsin and this relationship was eventually used to calculate a K_d of 4mM for NAD^+ and to demonstrate the ability to bind to the A_1 chain of one of the arginine model substrates.

Heat labile toxin (LT) was purified from *E.coli* 2107E (which carries the plasmid EWD299 ($LTA^+B^+Ap^r$)) using gel filtration on agarose and elution of pure LT wih galactose. The LT, which catalyses the same reaction as cholera toxin, was compared with

cholera toxin and found to have lower activity in an ADP-ribosylation assay.

The active site of the cholera toxin's A_1 chain was studied by photoaffinity labelling with underivatised NAD^+. The NAD^+ became covalently linked to the A_1 chain in a saturable manner. A peptide containing tritiated NAD^+ was purified from a tryptic digest of radiolabelled A_1 chain and its amino acid content determined by amino acid analysis. The amino acid sequence of the peptide could not be determined using a gas-phase sequencer based on reaction of the amino acids with phenylisothiocyanate neither could its amino terminal residue be detected using dansyl chloride. Fast-atom-bombardment mass spectrometry suggested a possible molecular weight of 1147 which together with the amino acid analysis allowed a tentative identification of the peptide as Asn·Asp·Asp·Lys corresponding to the first four residues of the A_1 chain with [4-^{3}H] NAD^+ attached to one or more of its residues.

Chapter 1 Introduction

Vibrio cholerae can produce one of the most profound physiological responses known to occur in any human infection. Without invading the body and without bringing about significant structural changes in any tissue, these bacteria can cause the host to become depleted of extracellular fluid to the point of collapse and death within a few hours. Fluid and electrolyte deficits result from a massive loss of diarrhoeal fluid (Sack, 1980). Clinically, this is manifest by vomiting, poor skin turgor, sunken eyes, deep breathing and intense peripheral vaso-constriction. This latter symptom is one of the most common causes of death (De, 1961). In untreated patients with clinical cholera the mortality rate may approach 70%, making this one of the most lethal of all bacterial diseases.

As is now known, these dramatic effects are due to a protein exo-enterotoxin called cholera toxin. Following ingestion of *Vibrio cholerae* in contaminated drinking water, the small intestine becomes infected and colonised with the bacteria which bind tightly to the mucosa. The bacteria then secrete cholera toxin into the lumen. At least a part of the toxin then enters the epithelial cells where it causes increases in cyclic AMP concentrations. This in turn alters the normal secretory processes of the small intestine by inhibiting uptake of sodium and chloride by the villus cells as well as by stimulating active anion secretion by the crypt cells (Field, 1980).

Vibrio cholerae was identified as the causative agent of cholera as long ago as 1854 by Pacini (see Pollitzer, 1965) but it

was not until 1959 that De, (1959) and Dutta *et al* ., (1959) convincingly demonstrated the existence of a cholera toxin. They observed that fluid accumulation in rabbit small intestine could be produced by cell-free culture filtrates of *V.cholerae*. Finkelstein and LoSpalluto, (1969) and later Richardson *et al*., (1970) purified the protein and showed it to be composed of several subunits. Field (1971) showed that cholera toxin greatly stimulated the flow of electrolytes across ileal mucosa, an event mimicked by cyclic AMP or substances such as theophylline that increases cyclic AMP concentrations (Field, 1980). It was proposed that cholera toxin activated adenylate cyclase and that the subsequent rise in cyclic AMP levels led to the change in flow of electrolytes. This idea was soon supported by a number of workers (Schafer *et al*., 1970; Kimberg *et al*., 1971; Sharp and Hynie, 1971). The observation that cholera toxin could induce lipolysis in fat cells in a cyclic AMP-dependant manner (Vaughan *et al*., 1970) suggested that the activation of adenylate cyclase was the primary action of cholera toxin and that it could be demonstrated not only in intestines but also in other cells and tissues.

The currently accepted view of the molecular mechanism of cholera toxin can be summarised as follows. The toxin consists of one A subunit and five B subunits. The B subunits can bind to ganglioside G_1, a natural toxin receptor on the cell surface. This causes a conformational change leading to the entry of all or part of the A subunit into the cell. During or after entry the A subunit is split into two chains, A_1 and A_2. The A_1 chain then catalyses the ADP-ribosylation of N_s, the stimulatory regulatory component of

the adenylate cyclase complex. ADP-ribosylation inhibits the GTP'ase activity of N_s, leaving it in a permanently activated state. The over-production of cyclic AMP generates a variety of physiological responses that vary with cell type (Gill and Meven, 1978; Cassel and Pfeuffer, 1978; Johnson *et al.*, 1978; Cassel and Selinger, 1977; Field, 1980). The biochemical aspects of cholera toxin have been extensively reviewed (see for instance van Heyningen 1977a, 1982b; Gill, 1982; Holmgren and Lönnroth, 1980; Gill and Enomoto, 1980, Moss and Vaughan, 1979; Lai, 1986).

1.1 The structure of cholera toxin

The complete toxin molecule is a protein of molecular weight 82,000 - 84,000 (van Heyningen, 1976; Lospalluto and Finkelstein, 1972; Lai *et al.*, 1976; Sattler *et al.*, 1975) and the purified toxin contains no detectable quantities of lipid or carbohydrate (Lospalluto and Finkelstein, 1972). Analysis of cholera toxin by SDS-PAGE (polyacrylamide gel electrophoresis in the presence of SDS) showed that the toxin contains two kinds of non-covalently associated subunits, called A and B (Finkelstein *et al.*, 1972; Cuatrecasas, 1973; Lönnroth and Holmgren, 1973). The toxin contains one A subunit (28,000) and several identical B subunits (8,000 - 14,000) (Finkelstein *et al.*, 1974; van Heyningen, 1974; Kurosky *et al.*, 1977; Gill 1976a). The B subunits exist as an aggregate even in the presence of SDS owing to very strong non-covalent interactions between them and can only be separated by boiling in SDS. The A subunit consists of two polypeptide chains called A_1 (22,000) and A_2 (6,000) connected by one disulphide bond. The A_1 and A_2 chains have been shown to be formed by post-translational modification by

nicking of a precursor polypeptide with bacterial proteases (Gill and Rappaport, 1979; Mekalonos *et al.*, 1979b). The protease responsible in *V.cholerae* is thought to be a soluble hemagglutinin protease (Booth *et al*, 1983). The "un-nicked" form of the toxin, produced by a protease-deficient strain of *V.cholerae* had greatly reduced enzyme activity (Mekalonos *et al.*, 1979b). The nicking of the A subunit does not produce any significant decrease in the molecular weight suggesting that few amino acids, if any, are lost. The precursor is believed to have the sequence NH_2-A_1-A_2-COOH (Duffy *et al.*, 1981).

1.2 Subunit arrangement

A molar ratio of five B subunits to every A subunit was proposed by Lai (1977) from calculatios of molar ratios using the molecular weights of each subunit and of whole toxin. This ratio has been verified (van Heyningen, 1977b; Gill, 1977). Some electron micrographs support this model (Ohtomo *et al.*, 1976) as do cross-linking experiments with bifunctional reagents (Gill, 1976b). X-ray diffraction analysis of crystalline toxin was consistent with five B subunits per molecule (Sigler *et al.*, 1977). Intact cholera toxin therefore consists of subunit A, comprising A_1 and A_2 chains, joined non-covalently to five B subunits, all associated non-covalently with each other. A heterologous ring of B subunits with subunit A in the centre is the proposed structure and is shown in fig. 1-1. The A_2 chain is thought to serve as a direct link between the A_1 chain and the B ring (Gill 1976a; van Heyningen, 1976; 1977b).

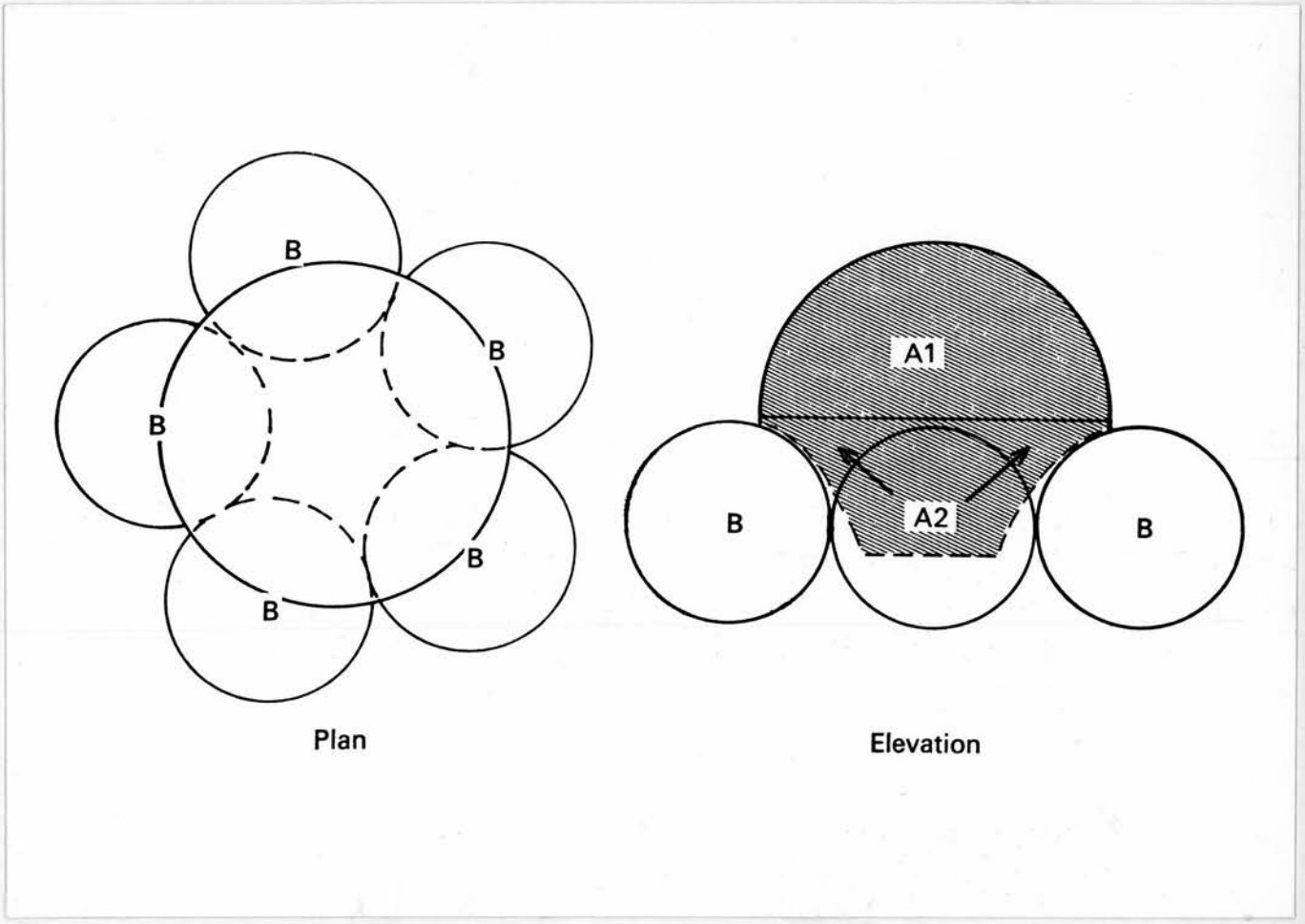


fig. 1-1 Proposed structural relationship of the cholera toxin subunits.

Taken from van Heyningen, 1977a

1.3 Primary structure of the subunits and the organisation of their structural genes

The primary structure of both subunits is known (Lai, 1977; Duffy *et al.*, 1981; Lai *et al.*, 1979). The B subunit contains 103 amino acid residues and shows significant similarity to the B chains of glycoprotein hormones such as thyrotropin, luteinizing hormone and follicle stimulating hormone (Ledley *et al.*, 1976; Kurosky *et al.*, 1977). The A subunit is unusually rich in charged residues (Duffy *et al.*, 1981). Before 1981 it was prohibited by the Guidelines for Recombinant DNA research to clone the genes encoding cholera toxin. Recognition of the structural and functional homology between E.coli heat labile toxin (LT, see Chapter 4) and cholera toxin was one of several reasons why the Recombinant DNA Advisory Committee voted to allow the cloning of cholera toxin genes at a suitable level of containment.

The toxin structural genes *ctx* A and *ctx* B have been cloned and their complete nucleotide sequence determined (Mekalonos *et al.*, 1983; Pearson and Mekalonos, 1982). The two genes are cotranscribed from a polycistronic operon (Mekalonos *et al.*, 1983) which in turn is part of a larger 7- to 12- kilobase genetic element (Mekalonos, 1983). Analysis of the nucleotide sequence of the ctx genes suggests that they are cotranscribed from a single promoter preceding the *ctx* A gene (Rosenberg and Court, 1979) and that *ctx* mRNA contains two Shine-Dalgarno sequences (Steitz, 1979) preceding the AUG translation starts for *ctx* A and *ctx* B. The Shine-Dalgarno sequence preceding B can potentially pair with the complementary sequence at the 3'- end of 16s-rRNA with greater efficiency than

that for the A cistron (Yamamoto *et al.*, 1984; Gennaro and Greenaway, 1983). Greater translational efficiency would then explain the production of five B subunits for each A subunit.

It appears that several genetic factors influence the level of cholera toxin production. The first is the copy number of the toxin genetic element; amplification of this element increases the production of toxin (Mekalonos, 1983), Secondly, the number of tandem repeats of a sequence T-T-T-T-G-A-T upstream of the ctx promoter affects toxin expression (Miller and Mekalonos, 1984). Lastly, there are at least two regulatory genes, one whose product stimulates transcription of *ctx* AB and one which may encode a negative regulatory factor (Miller and Mekalonos, 1984). Studying the factors involved in the control of cholera toxin production including nutritional and physiological factors may help to define the precise role that the toxin plays in pathogenesis because the intestine apparently provides a selective environment for bacterial cells which have the capacity to produce cholera toxin (Dutta and Habbu, 1955; Dutta *et al.*, 1963; Finkelstein, 1973).

1.4 Binding cells and ganglioside

The evidence that ganglioside G_{M1} is the receptor for cholera toxin is impressive (e.g. van Heyningen, 1974; Pierce, 1973; Moss *et al*, 1976a, 1977; Fishman, 1980). More than two million toxin molecules may bind to ganglioside G_{M1} located on the plasma membrane of each mucosal cell (Holmgren *et al.*, 1973). Binding is tight and irreversible (van Heyningen, 1977a). The pentavalent interaction

between subunit B and G_{M1} may lead to patching and capping of the toxin-ganglioside complex and induces changes in conformation in both B and A subunits (Mullin *et al.*, 1976; van Heyningen, 1982b). The binding of cholera toxin to cells is followed by a lag of from 15 to 90 mins. (Cuatrecasas, 1973; Gill and King, 1975; Bennet and Cuatrecasas, 1975; Fishman, 1980), depending on the type of cell, before the levels of adenylate cyclase inside the cell begin to rise. There was no lag when toxin was incubated with lysed or broken cells (van Heyningen and King, 1975) which suggested that the lag may have something to do with transport across the membrane or with release of A_1 from intact toxin. A general theory of the mechanism of entry of the toxin is summarised in fig. 1-2.

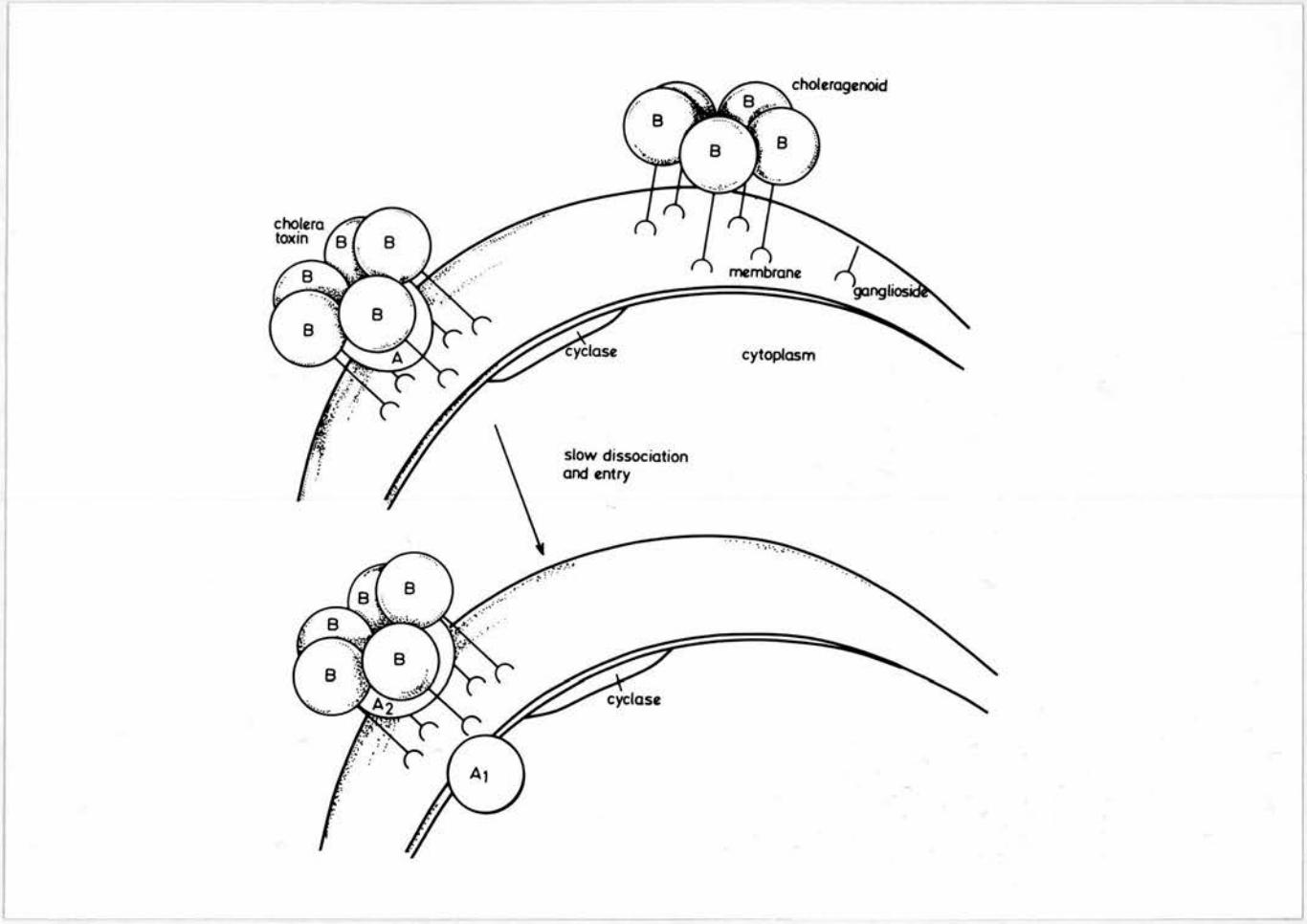


fig. 1-2 Model for the movement of the A_1 chain across the membrane.

The A_1 chain may remain associated with the rest of the toxin or may become completely dissociated. From van Heyningen, (1982b)

1.5 Entry of the A_1 chain

Adenylate cyclase is located on the inner surface of plasma membranes. To activate the cyclase, all or part of subunit A must be transported at least to the cytoplasmic surface of the cell membrane. This entry process is incompletely understood and several theories offer explanations. Gill (1976b) proposed that the B subunits form a channel through which the A subunit can pass although this theory is now not thought to be true. Wisnieski and Bramhall (1981) used photolabelling to show that only the A_1 subunit enters the membrane. The simplest theory originally suggested by van Heyningen and King in 1975 was based on their observation that subunit A was active alone even with intact cells, although at low specific activity. There may be no specific mechanism of entry, but the high local concentration of toxin following the binding to G_{M1} could increase the chance of a few molecules entering by some random process. As little as one molecule per cell may be all that is needed for activation of adenylate cyclase.

It is not clear whether the whole of the A_1 chain has to penetrate the membrane as quite small proteolytic fragments are claimed to show activity (Matuo *et al.*, 1976; van Heyningen and Tait, 1980). Van Heyningen (1977b) reported that cross-linked cholera toxin as well as native toxin could activate adenylate cyclase in intact cells. This suggests that the A_1 chain does not necessarily dissociate from the rest of the toxin as is passes through the membrane. Although some cells take up the whole toxin (such as neuronal cells (Joseph *et al.*, 1978)) most people assume that it remains outside the cell (Wisnieski and Bramhall, 1981).

1.6 Activation of adenylate cyclase

Cuatrecasas and co-workers proposed a model in which cholera toxin directly interacted with adenylate cyclase to activate it stoichiometrically (Bennet *et al.*, 1975; Sahyoun and Cuatrecasas, 1975). However, kinetic data suggested that the toxin acted enzymically and Gill (1975) proposed that cholera toxin caused an enzymic modification to adenylate cyclase or a regulatory component. NAD^+ had been found essential for cholera toxin's action in all cell-free systems examined (e.g. Bitensky *et al.*, 1975; Martin *et al.*, 1977). Moss *et al.*, (1976b) then discovered that cholera toxin catalysed the hydrolysis of NAD^+ to ADP-ribose and nicotinamide (NAD^+ Glycohydrolase activity, EC 3,2,2,5). Moss and Vaughan (1977) further demonstrated that the release of nicotinamide from NAD^+ was greatly stimulated by arginine. ADP-ribosyl—arginine was formed as a product.

Although much of the NAD^+ Glycohydrolase activity of crude toxin may be due to another enzyme (Tait and van Heyningen, 1978) it is clear that both NAD^+ Glycohydrolase and ADP-ribosyl transferase activities are true properties of the toxin, specifically of its A_1 chain. Reported K_m values for NAD^+ were 3.6mM (Mekalonos *et al.*, 1979a) and 5mM (Moss *et al.*, 1979b).

1.7 ADP-ribosylation catalysed by the A_1 chain

The hormone-sensitive adenylate cyclase system is complicated, consisting of at least three types of protein embedded in the plasma membrane. Receptors communicate with a pair of homologous GTP-binding regulatory proteins, N_s and N_i, which in turn stimulate (N_s) or inhibit (N_i) the activity of a catalytic subunit, C.

The role of GTP in the regulation of adenylate cyclase was first recognised by Rodbell *et al.*, (1971) who used it to restore hormone sensitivity to adenylate cyclase in isolated membranes. Levinson and Blume (1977) observed that cholera toxin activation of adenylate cyclase was dependant on GTP, as well as NAD^+. The magnitude of activation was quantitatively similar to that obtained with 5'-guanylyl-imidodiphosphate (Gpp(NH)p) alone, a non-hydrolysable analogue of GTP. It was suggested that cholera toxin was interfering with hydrolysis of GTP at some regulatory site and that this resulted in permanent activation of adenylate cyclase (Levinson and Blume, 1977; Schramm and Rodbell, 1975). Direct evidence for this model came from Cassel and Selinger (1977) using turkey erythrocyte membranes. A catecholamine-sensitive GTP'ase activity was observed in these membranes with properties suggesting it was associated with a GTP regulatory site of adenylate cyclase. Treatment of the membrane with cholera toxin, NAD^+, ATP and cytosol resulted in a decrease in hormone stimulated GTP'ase activity which paralleled the elevation of adenylate cyclase activity in these membranes. Thus toxin activation of adenylate cyclase was associated with an inhibition of GTP'ase activity. The guanyl nucleotide regulatory site of adenylate cyclase was presumably the site of both GTP dependant activation of cyclase and the inhibition of GTP'ase activity by cholera toxin, although separate sites for these activities have more recently been proposed (Gill and Woolkalis, 1985).

Pfeuffer (1977) first reported the purification of a GTP-binding protein from pigeon erythrocyte membranes which could be ADP-

ribosylated by cholera toxin and which could restore activation of adenylate cyclase to membranes depleted of their intrinsic GTP-binding proteins. The protein, of molecular weight 42,000 now seems to be a subunit of N_s (Katada and Ui, 1982). The homologous protein, N_i, has been shown to have intrinsic GTP'ase activity. A similar activity for N_s has not been purified but may be lost during purification (Enomoto and Asakawa, 1984). The two guanine-nucleotide-binding regulatory components, N_s and N_i, have been purified to apparent homogeneity (Bokoch *et al.*, 1983, 1984; Codina *et al.*, 1983, 1984; Sternweis *et al.*, 1981) and they appear to be structurally and functionally very similar. Both are $\alpha\beta\gamma$ heterotrimers (Hildebrant *et al.*, 1984a) of molecular weight 80,000-90,000 (Bokoch *et al.*, 1984; Codina *et al.*, 1984). Although their α subunits are unique, their β subunits (molecular weight 35,000) appear to be indistinguishable although apparent size heterogeneity has been reported (Sternweis and Robishaw, 1984; Codina *et al.*, 1984; Manning and Gilman, 1982). They can sequester and inactivate the α subunits. The role of the recently discovered γ subunit (molecular weight 5,000) is unknown. The α subunits of both proteins bind GTP (Northup *et al.*, 1982). Activation of either protein is guanine nucleotide- and Mg^{2+}-dependant (Northup *et al.*, 1982; Hildebrandt and Birnbaumer, 1983), is also brought about by NaF in the presence of Mg^{2+} (Sternweis *et al.*, 1981) and appears to result in the dissociation of the proteins into separate α and $\beta\gamma$ components (Hildebrant *et al.*, 1983; Bokoch *et al.*, 1983; Northup *et al.*, 1983). N_s and N_i are substrates for NAD^+-dependant ADP-ribosylation by bacterial toxins. The α subunit of N_s (molecular

weight 45,000) is ADP-ribosylated by cholera toxin; inhibiting its GTP'ase activity and prolonging its active state (Cassell and Selinger, 1977). The α subunit of N_i (molecular weight 41,000) is ADP-ribosylated by pertussis toxin. This inhibits GTP'ase activity presumably by stabilising the $\alpha\beta\gamma$ complex (Murayama and Ui, 1984; Katada *et al.*, 1984).

One theory of the sequence of events surrounding hormonal stimulation of adenylate cyclase is as follows (Gilman, 1984). The binding of an external signal to the receptors R_s and R_i (see fig. 1-3) induces a conformational change in the receptors which is transmitted to N_s and to N_i, making them susceptible to GTP. When the GTP binds it forces them into a different conformation and leads to dissociation of the subunits. N_α can then activate the catalytic subunit while N_i inhibits it. When the GTP'ase activity of these subunits has hydrolysed the bound GTP to GDP they re-associate into oligomers and return to the inactive state. The role of N_i in inhibiting the catalytic subunit is uncertain. N_α may simply release $N_{i\beta}$ which binds to $N_{s\alpha}$ and antagonises stimulation (Gilman, 1984) or it may act directly on the catalytic subunit without affecting the activity of N_s (Hildebrand *et al.*, 1984b; Enomoto and Asakawa, 1984). The effects of cholera toxin are then simply explained by its ability to prolong the life of the activated $N_{s\alpha}$-GTP complex to that the cell produces cyclic AMP continually even in the absence of an external signal calling for its manufacture. (fig. 1-4)

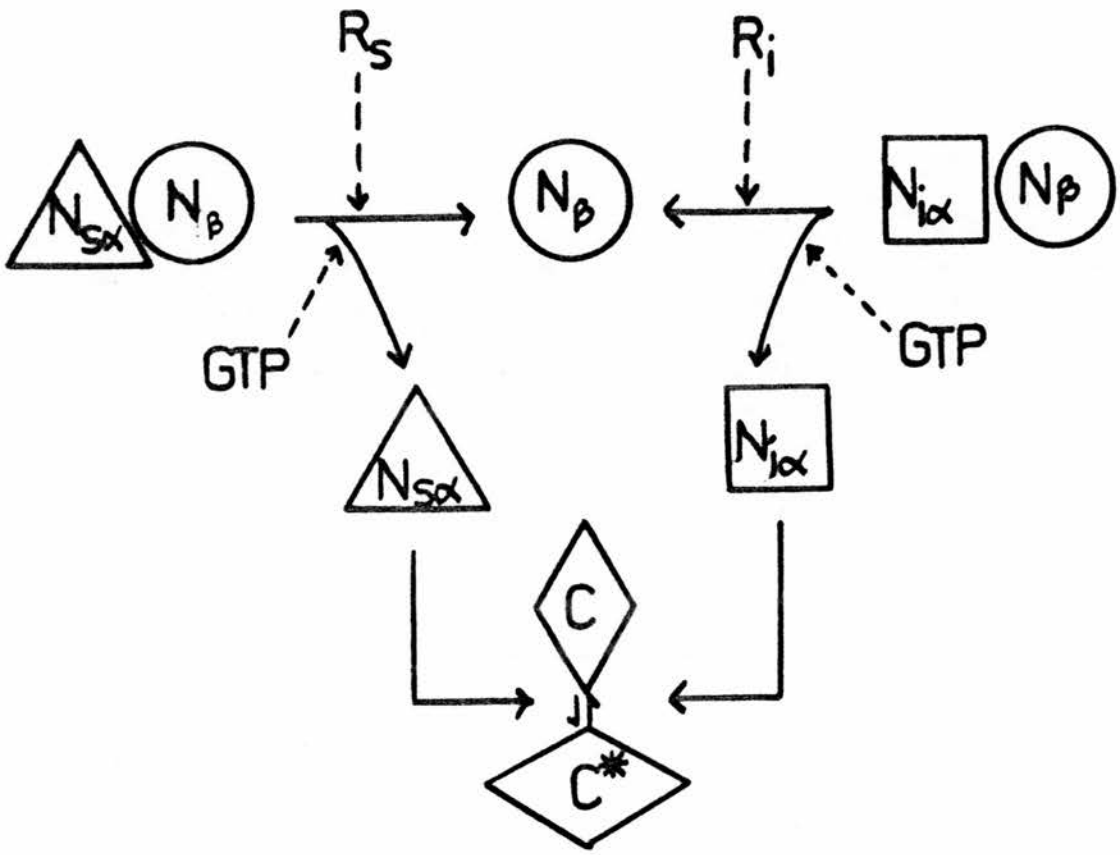


fig. 1-3 Schematic diagram of the interaction of the components of the adenylate cyclase complex.

$C_{\pm}$ is the catalytic component of adenylate cyclase, C^{*} denoting its active form from Gilman, 1984.

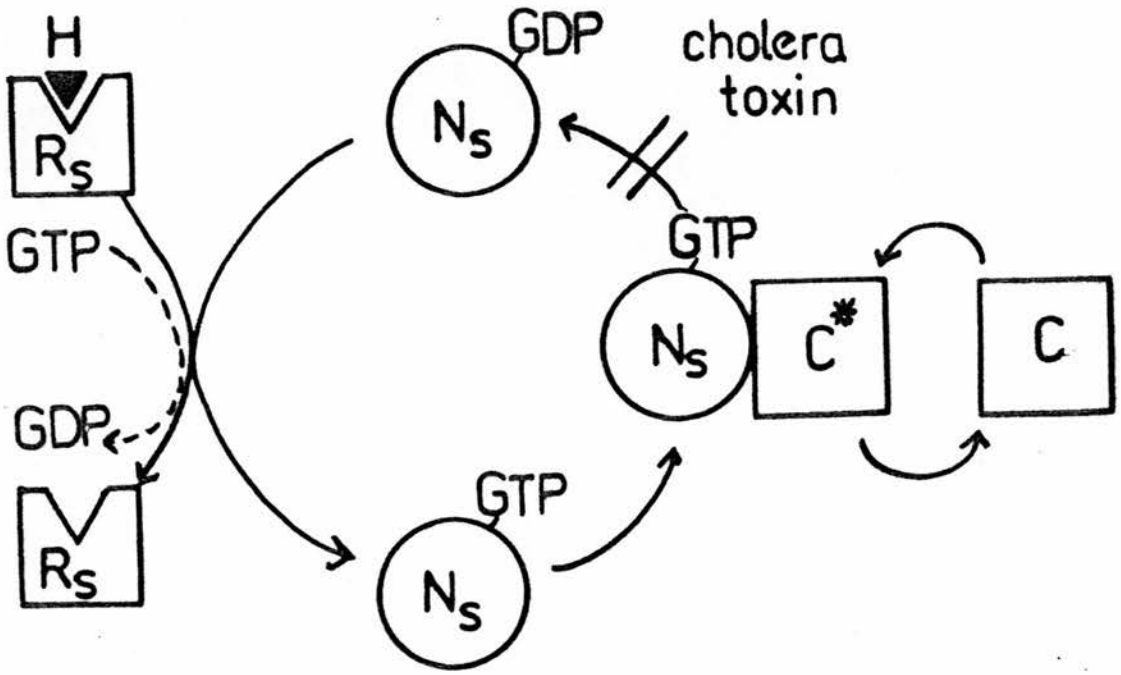


fig. 1-4 Simplified scheme showing the mechanism of activation of adenylate cyclase by cholera toxin.

R_s represents the stimulatory hormone receptor and H a stimulatory hormone (such as ACTH).

1.8 Other effects of the toxin

Although the ADP-ribosylation of N_s is thought to be responsible for the physiological effects of cholera toxin, it is not the only effect seen. In many tissues a protein of molecular weight 54,000 is also modified (Cooper *et al.*, 1981). Although peptide analysis of this protein indicates that it is closely related to the $N_{s\alpha}$ subunit (Manning and Gilman, 1983) and reconstitution studies implicate it in $N_{s\alpha}$ activity (Sternweis *et al.*, 1981; Hanski *et al.*, 1981) its role in the transmembrane signalling process is unclear. Owens *et al.*, (1985) report that cholera toxin may ADP-ribosylate the $N_{i\alpha}$ subunit under certain conditions causing similar functional consequences as ADP-ribosylation by pertussis toxin. It is not clear whether this reaction is significant *in vivo*. The toxin can certainly ADP-ribosylate several membrane-bound 'minor' substrates not all of which are likely to be larger precursors or proteolytic derivatives of N_s. Some have been identified, such as a major erythocyte membrane protein (Gill and Woolkalis, 1985). Soluble proteins, even globin, are ADP-ribosylated. Almost every abundant protein can be seen to be modified provided the detection is sensitive enough although some of these modifications may be non-enzymic, perhaps through the formation of Schiff's bases (Kun *et al.*, 1976). Whether these modifications cause any effects is uncertain, although cholera toxin has been reported to inhibit chemotaxis in a cyclic-AMP independant manner (Aksamit *et al.*, 1985) in human and rabbit neutrophils (Hill *et al.*, 1975; Bergman *et al.*, 1978), to block phosphate incorporation into pyruvate kinase (Tsuchoja, 1985) and decrease phosphate transport in canine renal

brush border membranes (Hammerman *et al.*, 1982). Again, the significance of these effects in the pathogenesis of *V.cholerae* is unclear. Recently, clinical trials have been undertaken in humans using an attenuated strain of *V.cholerae* as a live oral vaccine (Levine *et al.*, 1984). The strain used was Texas Star, an A^-B^+ mutant derived by nitrosoguanidine treatment from *V.cholerae* El Tor Ogawa strain 3083. Although the strain produced no A subunit it still caused some diarrhoea in 25% of cases. It is conceivable that the mere act of colonization of the small intestine may elicit some response (Smith and Linggood (1971)). It has also been claimed that *V.cholerae* can elaborate cytotoxins and other "toxic materials" distinct from cholera toxin that have the ability to induce secretion when tested in animal models (Nishibuchi and Seidler, 1983; Nishibuchi *et al.*, 1983; Sanyal *et al.*, 1983). These factors may become important when considering the production of oral vaccines based on attenuated strains of *V.cholerae* and indicate that the actual disease-state of cholera may be caused by a number of factors working together, of which the ADP-ribosylation of N_s is the most significant.

1.9 N_s as a model for other GTP-binding proteins

It is now apparent that N_s and N_i are not the only GTP-binding proteins which transduce and integrate information flow across the plasma membrane. A whole family of similar GTP-binding proteins, or G proteins has been proposed (Hughes, 1983; Berridge, 1985). Receptor molecules at the surface of the cell are thought to transmit information through the plasma membrane and into the cell through these G proteins; membrane proteins which apparently cannot

be active unless they bind GTP. In an analagous way to N_s and N_i, the G proteins are then proposed to act on enzymes which regulate the concentration of highly phosphorylated intracellular messengers such as cyclic GMP and inositol triphosphate. The functional homologies have been backed up in many cases by close structural homologies. Both cholera toxin and pertussis toxin (produced by *Bordetella Pertussis*) have been instrumental in the elucidation of some of these processes as they show the ability to ADP-ribosylate proteins homologous to their respective substrates, N_s and N_i. (see fig. 1-5).

The most striking structural and functional homology is that seen between N_s, N_i and transducin, a protein responsible for mediating rhodopsin-catalysed photoactivation of a cyclic GMP-phosphodiesterase (Yamazaki *et al.*, 1983; Fung, 1983; van Dop *et al.*, 1984). In a similar way to activation of N_s or N_i, photoactivation of rhodopsin (c.f. hormone-receptor interaction) leads to a conformational change in transducin which in turn leads to activation, GTP-binding and dissociation of its $\alpha\beta\gamma$ subunits (Fung and Stryer, 1980; Kuhn, 1980). The striking homologies between transducin, N_s and N_i are covered in more detail in section 3.2.9. Transducin is a substrate of both cholera toxin and pertussis toxin.

A number of other G-proteins have been reported, about which less is known. a GTP-binding protein was identified in brain tissue in 1984 (Sternweis and Robishaw, 1984; Neer *et al.*, 1984). The protein, designated N_o was present in high concentrations and was a substrate for pertussis toxin. It has a similar subunit structure

G-protein	subunit composition	molecular mass (kDa)	factors mediating guanine nucleotide exchange	ADP-ribosylation by bacterial toxins
N_s	$\alpha_1\beta\gamma$	45 35 10	hormone-receptor complex	cholera toxin
N_i	$\alpha\beta\gamma$	41 35 10	hormone-receptor complex	pertussis toxin
transducin	$\alpha\beta\gamma$	39 35 10	photoactivated rhodopsin	cholera and pertussis toxins
N_{in}	$\alpha\beta\gamma$	25	unknown	cholera toxin
N_o (brain)	$\alpha\beta\gamma$	39 35 11	unknown	pertussis toxin
p21	α?	21	unknown	-

fig. 1-5 Proposed G-protein family

to N_s and N_i with subunits of similar sizes (Milligan and Klee, 1985; Malbon *et al.*, 1984). N_o has not been found in other tissues and its function is unknown.

Another novel G protein may be involved in coupling hormone receptors to phospholipase C, an enzyme which converts phospholipids in the plasma membrane into the intracellular messengers inositol triphosphate and 1,2 diacylglycerol (for recent reviews see Williamson *et al.*, 1985 and Majerus *et al.*, 1985). The protein has been tentatively named N_p by Cockroft and Gompert (1985) and is a substrate for pertussis toxin (Nakamura and Ui, 1985; Brandt *et al.*, 1986). N_p perhaps represent a particular permutation of the subunits of N_i in which phosphorylation of the B subunit (stimulated by epidermal growth factor) has altered its function to allow it to interact with phospholipase C (Joseph, 1985).

It has also been proposed (Heyworth *et al.*, 1983) that a specific G-protein might mediate the effect of insulin on plasma membrane kinases and phosphodiesterases. The proposed N_{in} is suggested (Heyworth *et al.*, 1985) to have a protein component of molecular weight 25,000 which has a GTP-binding site, a site for cholera toxin-mediated ADP-ribosylation and a site for interaction with the insulin receptor. Insulin could inhibit ADP-ribosylation of N_{in} by cholera toxin.

An analogy can also be drawn between the G-protein family and p21 proteins. These are a highly related group of proteins of 189 amino acids (and hence molecular weights around 21,000) encoded by the multigene family of mammalian *ras* oncogenes (Change *et al.*, 1982; McGrath *et al.*, 1983; Taparowsky *et al.*, 1983). The proteins have reported amino acid homology to both transducin and GTP-binding elongation factors (Lochrie *et al.*, 1985). It is of note that the homologies to transducin are confined to regions implicated in the binding and hydrolysis of GTP. *In vitro*, p21 proteins bind GTP and are membrane bound although their function is unknown (Scolnick *et al.*, 1979; Papageorge *et al.*, 1982; Finkel *et al.*, 1984). Normal and mutant transforming *ras* proteins show no alteration in localisation or GTP-binding but the intrinsic GTP'ase activity of normal *ras* proteins is tenfold lower in transforming mutants (Sweet *et al.*, 1984). It is tempting to speculate on the functional analogy between oncogenic *ras* proteins and N_s and N_i. In the latter, ADP-ribosylation inhibits GTP'ase activity and leaves N_s and N_i permanently switched on. Perhaps the transforming mutations which destroy the *ras* protein's GTP'ase activities leaves them in a

'switched-on' form, which leads in some unknown way to the over-proliferation characteristic of tumour cells. If the analogy were to hold then suitable effectors and receptors for *p21* would have to be found. A candidate for the receptor might be a peptide growth factor, despite the lack of evidence (Bourne, 1985). Phospholipase C has been mentioned as a possible effector (Smith, 1985). However, despite the presumed similarities, Gill and Woolkalis (1985) could not detect any ADP-ribosylation of *p21* by cholera toxin. Further biochemical and genetic analysis of this and other systems may yet identify other proteins involved in the transduction of information. The ease of purification of transducin (Kuhn, 1980) and N_o (Sternweis and Robishaw, 1984) and the use of cholera toxin as a tool in highlighting similar protein structures and functions and in labelling proteins using (^{32}P)-NAD$^+$ may allow a greater understanding of how these systems function.

1.10 Other ADP-ribosylation reactions

ADP-ribosylation is not a reaction unique to cholera toxin. As shown in fig. 1-6, it is shared by other bacterial toxins, by bacteriophages and even by certain eukaryotic enzymes.

E.coli heat-labile toxin

Of the ADP-ribosylating bacterial toxins, *E.coli* heat-labile toxin (LT) shows the most striking similarities to cholera toxin. LT is produced by certain entero-pathogenic strains of *E.coli* and induces diarrhoea in exactly the same way as cholera toxin, namely by catalysing the ADP-ribosylation of a susceptible arginine on N_s (Evans *et al.*, 1972; Gill *et al.*, 1981; Moss and Vaughan, 1981). The two proteins are highly homologous (Spicer and Noble, 1982;

Dykes *et al.*, 1985) and there seems little doubt that they are evolutionarily related.

Diphtheria toxin

Diphtheria toxin shares with the A exotoxin from *P.aeruginosa* the ability to catalyse the ADP-ribosylation of elongation factor 2 (EF-2), a protein essential for ribosomal protein synthesis (Honjo *et al.*, 1968; Iglewski and Kabort, 1975; van Ness *et al.*, 1980). Like N_s, EF-2 is a GTP'ase and ADP-ribosylation destroys this activity, preventing protein synthesis and leading to cell death (Honjo *et al.*, 1968). However, these two proteins differ from cholera toxin in that they catalyse the ADP-ribosylation not of arginine but of diphthamide, a modified histidine so far found only in EF-2 (van Ness *et al.*, 1980) (see fig. 1-7).

Enzyme	Protein	Amino acid	Reference
Prokaryotes			
Diphtheria toxin	Ef-2	diphthamide	
P.aeruginosa exotoxin A	Ef-2	diphthamide	
T4 phage *mod* and *alt* proteins	*E.coli* RNA polymerase σsubunit and other proteins	arginine	Goff (1974) Rohrer *et al.*, (1975)
cholera toxin	N_s and other proteins	arginine?	
E.coli LT	N_s and other proteins	arginine?	Tait *et al.*, (1980)
Pertussis toxin	N_i and other proteins	asparagine, aspartate or cysteine	
P.aeruginosa exotoxinS	Ef-1 and associated proteins	unknown	Iglewski *et al.*, (1978)
N_A phage	*E.coli* proteins	unknown	Pesce *et al.*, (1976)
Eukaryotes			
mammalian cytosolic enzyme	Ef-2	dipthamide	
avian erythrocyte enzyme	N_s (?), soluble proteins	arginine?	
mammalian cytoplasmic enzyme	soluble proteins	arginine?	Moss and Stanley, (1981)
mammalian membrane enzyme	N_s, membrane proteins	arginine?	Beckner and Blecher., (1981) De Wolf *et al.*, (1981)
mammalian mitochondrial enzyme	mitochondrial inner membrane protein	arginine?	Adamietz *et al.*, (1981)
avian nuclear enzyme	nuclear proteins	arginine?	Tanigawa *et al.*, (1984)

fig. 1-6 Other ADP-ribosylation reactions

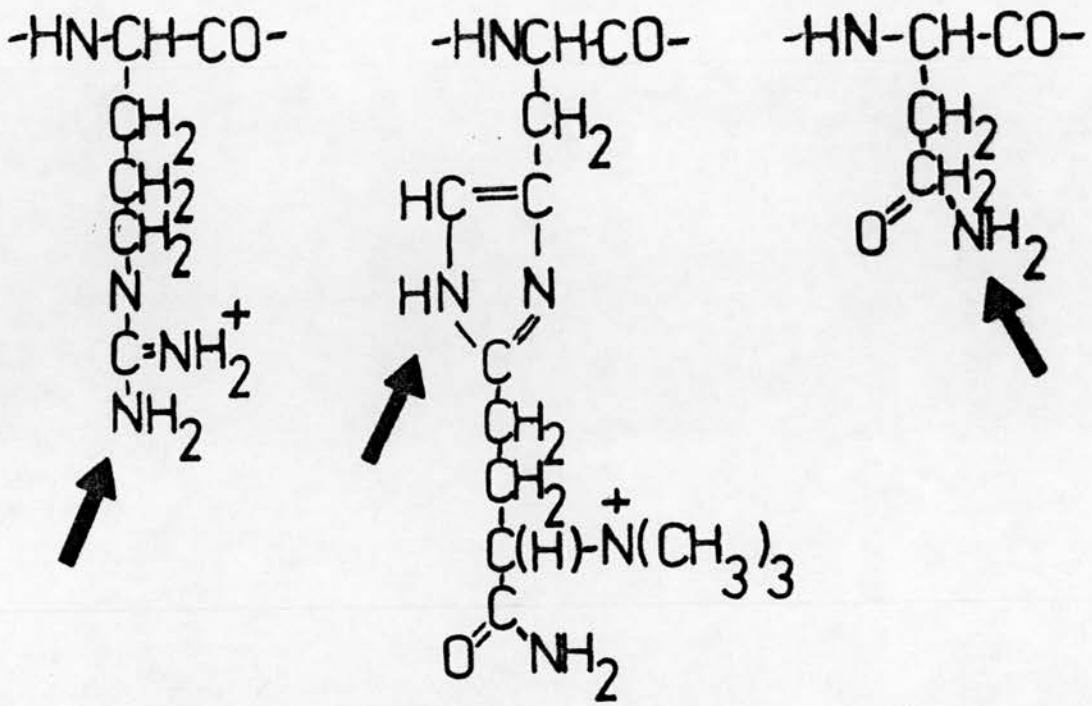


fig. 1-7 **Acceptor amino acids for ADP-ribosylation**

Diphthamide (2-[3-carboxyamido-3(trimethyl amino) propyl] histidine is formed by post-translational modification of a histidine residue.
The acceptor amino acid for pertussis toxin may be asparagine (shown above), aspartate or cysteine

Pertussis toxin

Much less is known about pertussis toxin, also known as islet activating protein (Katada and Ui, 1982). It is a protein of complex structure (Manning *et al.*, 1984) which catalyses the ADP-ribosylation of N_i. The reaction has been studied using transducin which can also act as a substrate. The amino acid which is modified has been variously reported to be aspartate (Hurley *et al.*, 1984), asparagine (Abood *et al.*, 1982; Manning *et al.*, 1984) or cysteine (West *et al.*, 1985).

Mono (ADP-ribosyl) transferases in eukaryotic cells

Poly (ADP-ribosyl) transferases in the nucleus have been extensively studied (see Hayaishi and Ueda, (1985) for a recent review) and have been implicated in such processes as DNA strand repair, sister chromatid exchange and other processes involving DNA strand cleavage (Gaal and Pearson, 1985; Hayaishi and Ueda, 1982). In addition, a number of mono (ADP-ribosyl) transferases have been identified. Their function is, however, less clear. For instance an avian erythrocyte protein has been identified (Moss and Vaughan, 1978) which can apparently ADP-ribosylate N_s, resulting in stimulation of adenylate cyclase (Beckner and Blecher, 1981; De Wolf *et al.*, 1981; Vitti *et al.*, 1982). The protein is partially homologous to cholera toxin (Ledley *et al.*, 1976), is specific for arginine and appears to share aspects of the kinetic mechanism of cholera toxin (Osborne *et al.*, 1985). Another mono (ADP-ribosyl) transferase has been reported which catalyses the ADP-ribosylation of EF-2 at its diphthamide residue (Lee and Iglewski, 1984).

The existence of these supports the view that the eukaryotic

enzymes might be functioning as physiological counterparts of cholera toxin and diphtheria toxin. The former might presumably have a role in signal transduction and the latter in the control of protein synthesis (although it would have to be under very tight control to avoid cell death). It seems that ADP-ribosylation must play some important role in the eukaryotic cell judging from the large number of ADP-ribosyl transferases identified, although what that role is, is as yet unknown.

Chapter 2 Materials and Methods

2.1 Materials

Cholera toxin

Cholera toxin was obtained from either Sigma Chemical Co. or from List Biochemicals, Richmond, California. Samples of isolated cholera toxin A_1 chain were provided by the Institut Merieux, France.

Radioactive chemicals

[nicotinamide-4-^{3}H]-NAD^+ (1.01Ci/mMol) and [U-^{14}C]NAD^+ (1.0Ci/mMol) were from Amersham International. [Adenine-2,8-^{3}H]NAD^+ (27.1Ci/mMol) was from New England Nuclear.

Other materials and reagents

NAD^+ was Grade 1 from Boehringer Mannheim. Bovine serum albumen (BSA), alkaline phosphatase, trypsin, chymotrypsin, benzyol-δ-arginine ethyl ester (BAEE), 4-chloro-7-nitrobenzofuran (Nbf), thio-NAD^+, nicotinic acid adenine dinucleotide, deamide-NAD^+, 3-aminopyridine adenine dinucleotide (APAD), adenine, adenosine, ADPR, FAD, $NADP^+$, NADH, NMN, AMP, ADP and ATP were all from Sigma Chemical Co.

Bio-Gel P10 agarose (100-200mesh) and Bio-Gel A5M agarose were from Bio-Rad. Sephadex G75, Sephadex QAE-25 and Sephadex G25 were from Pharmacia Fine Chemicals.

Pre-coated aluminium backed sheets of silica gel (60F254, 5cm x 7.5cm) were from Merck. Polyamide sheets (15cm x 15cm) and dansyl-amino acid derivatives were from BDH.

Aminoguanidine bicarbonate and p-nitrobenzaldehyde were from Aldrich Chemical Co.

Spectrapor dialysis membrane was from Spectrum Medical Industries.

[12-[(phenylmethyl) amino] dodecanyl] guanidine, [10-[(phenyl methyl) amino] decanyl] guanidine and [10-(acetyl amino) decanyl] guanidine were made available as intermediates from synthetic programmes within Glaxo Group Research Ltd.

All other reagents were analytical grade.

General methods

2.2 Preparation of Cholera Toxin

Freeze-dried cholera toxin or *E.coli* heat labile toxin (LT) were rehydrated to give a 1-10 mg/ml solution in 50mM tris-HCl, 100mM sodium chloride, 3mM sodium azide, 1mM EDTA, pH7.5 (TEAN buffer).

Isolated A_1 chain was stored frozen. Aliquots were thawed as required and dialysed against 0.1M Glycine/HCl, 6.5M urea, 5mM DTT pH3.2. The dialysed solution was then applied to a column of Sephadex G75 (1cm x 110cm) and run at 10ml/hour (Moss *et al.*, 1976). Fractions of 1ml were collected and monitored by absorbance at 280nm. (A 10mg/ml solution of A_1 chain was assumed to have an absorbance of 7.6 at 280nm (Holmgren and Lönnroth, 1980)). The purity of the A_1 chain was checked by 12% SDS-PAGE. Urea was removed by gradual dialysis against solutions of TEAN buffer, pH7.5 containing stepwise urea concentrations decreasing from 6M to zero.

2.3 Separation and reconstitution of subunits

2.3.1 Isolation and assay of subunits

Subunits of cholera toxin and LT were separated by gel filtration on Sephadex G75 as described in section 2.2 except that DTT was omitted from the buffer. Alternatively the same G75 column was used but with 5% formic acid in place of urea buffer (Lai *et al.*, 1979). A typical elution profile is shown for cholera toxin in fig. 2-1. However, when LT was treated in this way, the subunits were incompletely separated and the process had to be repeated to allow complete separation. (fig. 2-1).

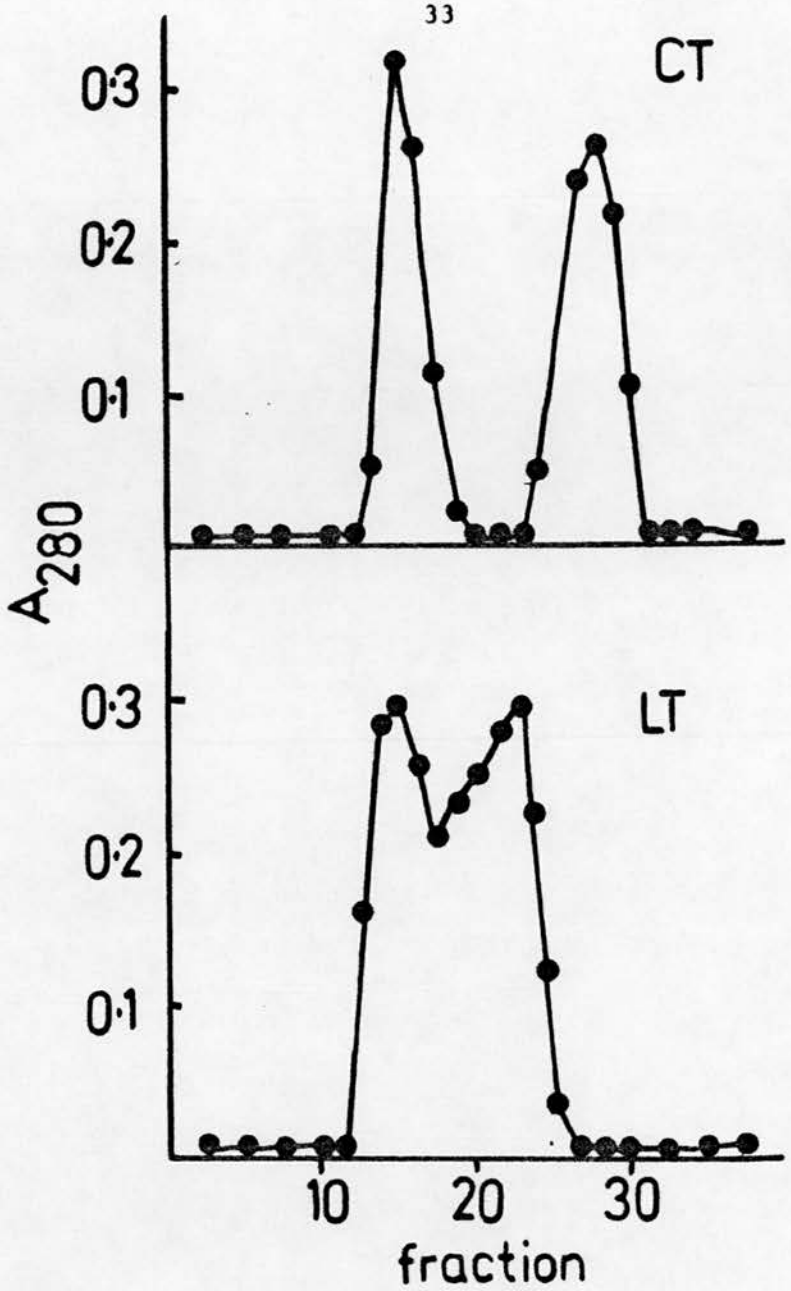


fig. 2-1. **Separation of cholera toxin and LT subunits by gel filtration chromatography**

The toxins, 5mg/ml in 6.5M urea, 0.1M Glycine pH3.2 were chromatographed on Sephadex G75 (1cm x 110cm) at 10ml/h. Fractions of 1.5ml were collected and monitored by absorbance at 280mm

The activity of subunit A was measured using an ADP-ribosylation assay described in section 2.9. Binding of subunit B to ganglioside was measured quantitatively by diffusion in agar (Holmgren *et al.*, 1973). Wells containing 6µl of solution and about 5mm apart were cut in a 1.5% agar gel made up in 50mM sodium phosphate, 150mM sodium chloride pH7.0. One well was filled with a solution of toxin or subunit (about 5µg/ml) and the others with ganglioside G_{M1} solution (about 1µg/ml). The plates were incubated at 4°C for 20 hours.

2.3.2 Reconstitution of separated subunits

Native toxin was reconstituted from subunits A and B by mixing solutions together in the presence of 8M urea and then dialysing the urea away as described. The two solutions were mixed so that the ratio (A_{280} of B)/(A_{280} of A) was 1.45. This ratio was calculated from the observed yield of columns used to separate the two subunits from native toxin, assuming that the yield of each subunit from the column was the same.

2.4 Purification of *E.coli* Heat Labile toxin

The method followed was essentially that of Hofstra *et al.*, (1984). The work described in this section was performed in the Department of Microbiological Biochemistry, Glaxo Group Research Ltd., Greenford, Middlesex. *E.coli* 2107E, a strain carrying the plasmid EWD299 (Amp^R, porcine LT^+) was grown in casamino acid growth medium containing Ampicillin for 8 hours at 37°C with vigorous shaking. This culture was then used to inoculate ten one litre flasks each containing 500ml growth medium. The flasks were incubated overnight at 37°C with vigorous shaking and the cells

harvested by centrifugation at 5000xg for 10 min. The cells were suspended in 50ml TEAN buffer, pH7.5 and were lysed by sonication (three one minute blasts). Cell debris was removed by centrifugation for one hour at 5000xg and the resulting supernatant subjected to ammonium sulphate precipitation at 60%. The precipitate was centrifuged at 2000xg for 10 min. and the pellet resuspended in TEAN buffer, pH7.5 before dialysis overnight against TEAN.

The protein was further purified on a column of Bio-Gel agarose A5m (2.5 x 90cm) pre-equilibrated with TEAN, pH7.5. The column was run at 20ml/hour and fractions of 5ml were collected. An initial wash with about 100ml TEAN buffer pH7.5 was continued until no more protein was eluted. The LT was then specifically eluted with 0.2M galactose. Throughout the purification procedure, samples were assayed for total protein, for the presence of LT using a G_{M1}-ELISA procedure described in section 2.5 and for ADP-ribosyl transferase activity using the assay described in section 2.9. LT purified in this way was analysed on 12% SDS=PAGE. Typically 20-25mg LT was recovered per ten litre culture.

2.5 Identification of *E.coli* heat labile toxin using a ganglioside immunosorbent assay (G_{M1}-ELISA) procedure

The method used was essentially that of Svennerholm and Holmgren, (1978). 96-well microtitre plates (Nunc) were coated with 50µl per well of a solution of phosphate-buffered saline (PBS)*. After 16 hours at 37°C, free ganglioside was removed by washing three times with PBS. Free binding sites were blocked with 100µl/well 2% (v/v) BSA in PBS, left for 30 min. at 37°C and the plates washed again

* containing 10µg/ml free ganglioside

with PBS. Standard LT solutions, or test samples, were added (50µl in PBS), incubated for one hour at 37°C and washed with PBS containing 0.05% (v/v) Tween 20. The presence of bound LT was determined by the sequential addition of 50µl rabbit anti-LT serum, 50µl goat anti-rabbit immunoglobulin alkaline phosphatase conjugate and 50µl p-nitro phenyl phosphate with incubation at 37°C for one hour and washing with PBS containing 0.05% Tween 20 between each step. The absorbance of each well was read at 405nm and the concentration of toxin determined from a standard curve shown in fig. 2-2. The optimum dilution for each batch of serum was determined from a dilution curve such as the one shown in fig. 2-3. In this case the optimum dilution was taken to be 1/200.

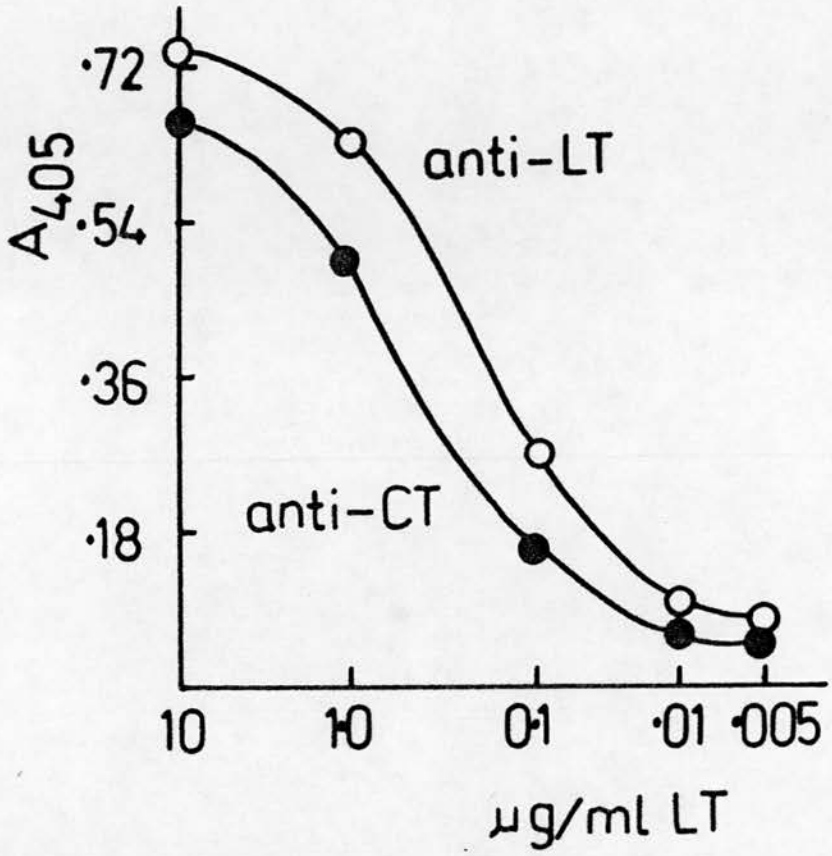


fig. 2-2 Standard curves of LT with anti-cholera toxin (anti-CT) and anti-LT antisera

LT purified as described in section 2.4 was diluted to cover the range 10 - 0.005 µg/ml. The antibody dilutions used were 1/50 for the anti-CT and 1/200 for the anti-LT (see fig. 2-3). The absorbance at 405nm of each toxin sample was determined following the G_{M1}-ELISA procedure described

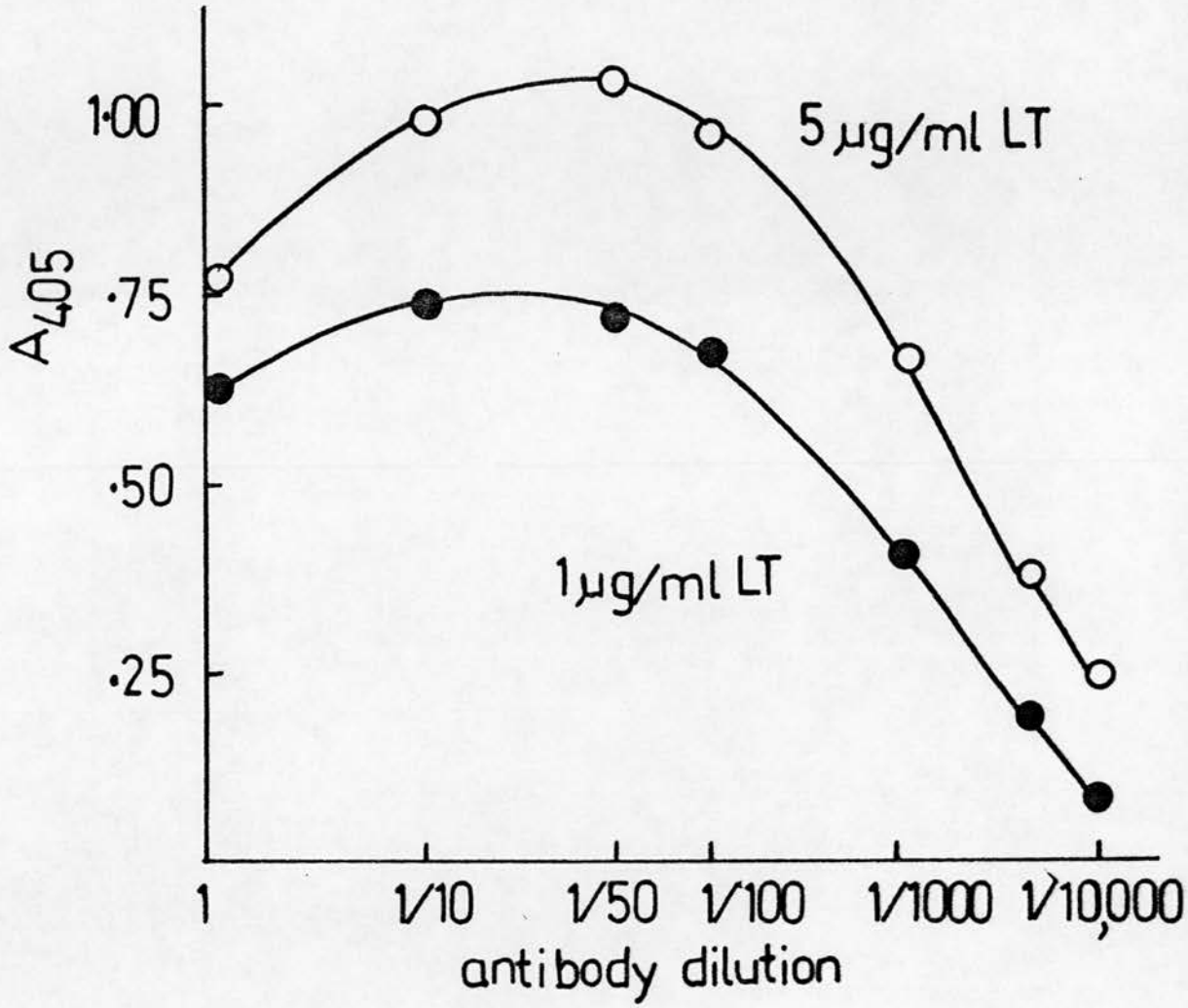


fig. 2-3 Antibody dilution curve

To determine the optimum dilution factor for each batch of antiserum, dilutions were made of up to 1/10,000 and tested using either 1µg/ml or 5µg/ml LT. The results are presented of absorbance of each sample at 405nm following the G_{M1} ELISA procedure described.

2.6 Purification of Transducin from Bovine Retinal Rod Outer Segments (ROS)

2.6.1 Bovine ROS membrane preparation

Transducin is one of several peripherally bound membrane proteins in the rod outer segments (ROS) of the eye. It is soluble in the dark in hypotonic buffer but becomes membrane bound upon illumination (Kuhn, 1980). This property has been exploited in the purification of transducin which can be specifically eluted from purified ROS membranes with GTP.

Fresh cattle eyes were obtained from a local slaughter house (Gorgie Abbatoir). Bovine ROS membranes were isolated in buffer solution containing 60mM KCl, 30mM NaCl, 2mM $MgCl_2$, 1mM DTT, 0.1mM PMSF and 10mM MOPS pH7.5 at 4°C. One hundred retinas were stirred gently in a beaker containing 30ml of 45% (w/v) sucrose in buffer for 3 min. The suspension was then diluted with an additional 100ml of 45% sucrose and centrifuged at 2445xg for 15 min. The supernatant was collected and diluted with an equal volume of buffer and recentrifuged at 43500xg for 15 min. The resulting crude ROS membranes were further purified by flotation centrifugation in 35% sucrose in buffer (Hong and Hubbell, 1973). Purified ROS membranes were washed twice with buffer, freeze-dried, and stored frozen at -20°C. When analysed by 10% SDS-PAGE the major protein band present in the purified ROS corresponded to a molecular weight of 38,000 representing rhodopsin, which makes up 80% of the total protein present in ROS. The rhodopsin concentration was determined in the presence of 0.1M tridecyl trimethyl ammonium bromide using a molar absorptivity of $42600 M^{-1}cm^{-1}$ at 500nm (Hong and Hubbell, 1973).

2.6.2 Purification of Transducin from Bovine ROS membranes

Transducin was isolated from ROS membranes by the method of Kuhn, (1980). Purified ROS from 500 retinas containing approximately 150 - 200mg rhodopsin were suspended in 80ml 5mM tris-HCl, 2mM DTT, 0.5mM $MgCl_2$, 0.05mM PMSF pH7.5 at 4°C and exposed to room light for 10 min. This bleaching causes certain polypeptides, among them transducin, to remain membrane bound. The membranes were then sedimented at 45000xg for 30 min. The pellet was washed twice more with buffer to remove phosphodiesterase. Transducin was then eluted by suspending the pellet in 50ml buffer containing 100µM GTP and centrifuging at 45000xg for 30 min. This extraction was repeated twice. The resulting solution containing 15 - 20mg transducin was freeze-dried and stored at -20°C. Transducin purified in this way was analysed by 10% SDS-PAGE.

2.6.3 Reconstitution of Transducin into ROS membranes

The method used was essentially that of Roof *et al.*, (1982). Membranes stripped of peripheral proteins were prepared by resuspending ROS membranes from 50 retinas in 5ml of 10mM NaCl, 0.25mM KCl, 1mM $CaCl_2$, 2mM $MgCl_2$, 5mM HEPES pH7.5 at 4°C. After two hours in the dark, the stripped ROS membranes were pelleted by centrifugation at 40000xg for 20 min. For reconstitution, freshly prepared stripped membranes were incubated with 300 - 400µl transducin (2.5mg/ml) in 100mM NaCl, 2mM $CaCl_2$, 5mM $MgCl_2$ and 10mM HEPES pH7.5 at 4°C and gently stirred overnight. The reconstituted membranes were diluted ten fold with 5mM HEPES solution and pelleted by centrifugation at 45000xg for 30 min., resuspended in 5mM tris-HCl, 2mM DTT, 0.5mM $MgCl_2$, 0.05mM PMSF and used at once.

2.7 Polyacrylamide Gel Elecrophoresis (PAGE)

2.7.1 General Method

Polyacrylamide gel electrophoresis was routinely conducted under denaturing conditions, i.e. in the presence of 0.1% (w/v) SDS (SDS-PAGE). The gel and buffer system was based upon that of Laemmli, (1970) with the addition of 2mM EDTA to chelate metal ions which may interfere with the polymerisation of acrylamide and cause aggregation of proteins (Douglas and Butow, 1976). Polyacrylamide of molecular weight up to 5 x 10^6 was added to 0.5% (w/v) in the separating gel to increase the mechanical strength. Protein samples (20 - 100µl) were mixed with 5µl 50% 2-mercaptoethanol in water, 5µl 10% SDS, 5µl 0.1% bromophenol blue and a drop of glycerol and incubated in a boiling water bath for one minute. Samples were applied to gels and electrophoresed at 100mV for about five hours. Gels were fixed in 20% methanol (v/v), 10% acetic acid (v/v) for 15 min., stained in 55% methanol (v/v), 10% acetic acid (v/v), 0.25% coomassie brilliant blue R (w/v), then destained until the gel background was clear in 5% methanol (v/v), 7.5% acetic acid (v/v). The staining procedure was carried out at 55°C.

For determination of molecular weights, gels were calibrated with the following standard proteins: αlactalbumin, soybean trypsin-inhibitor, trypsinogen, bovine erythrocyte carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin. Using these proteins, the standard curve shown in fig. 2-4 of electrophoretic mobility on 10% SDS-PAGE against the log of the molecular weight was obtained. This curve was subsequently used for molecular weight determination in the

range 10000 to 70000.

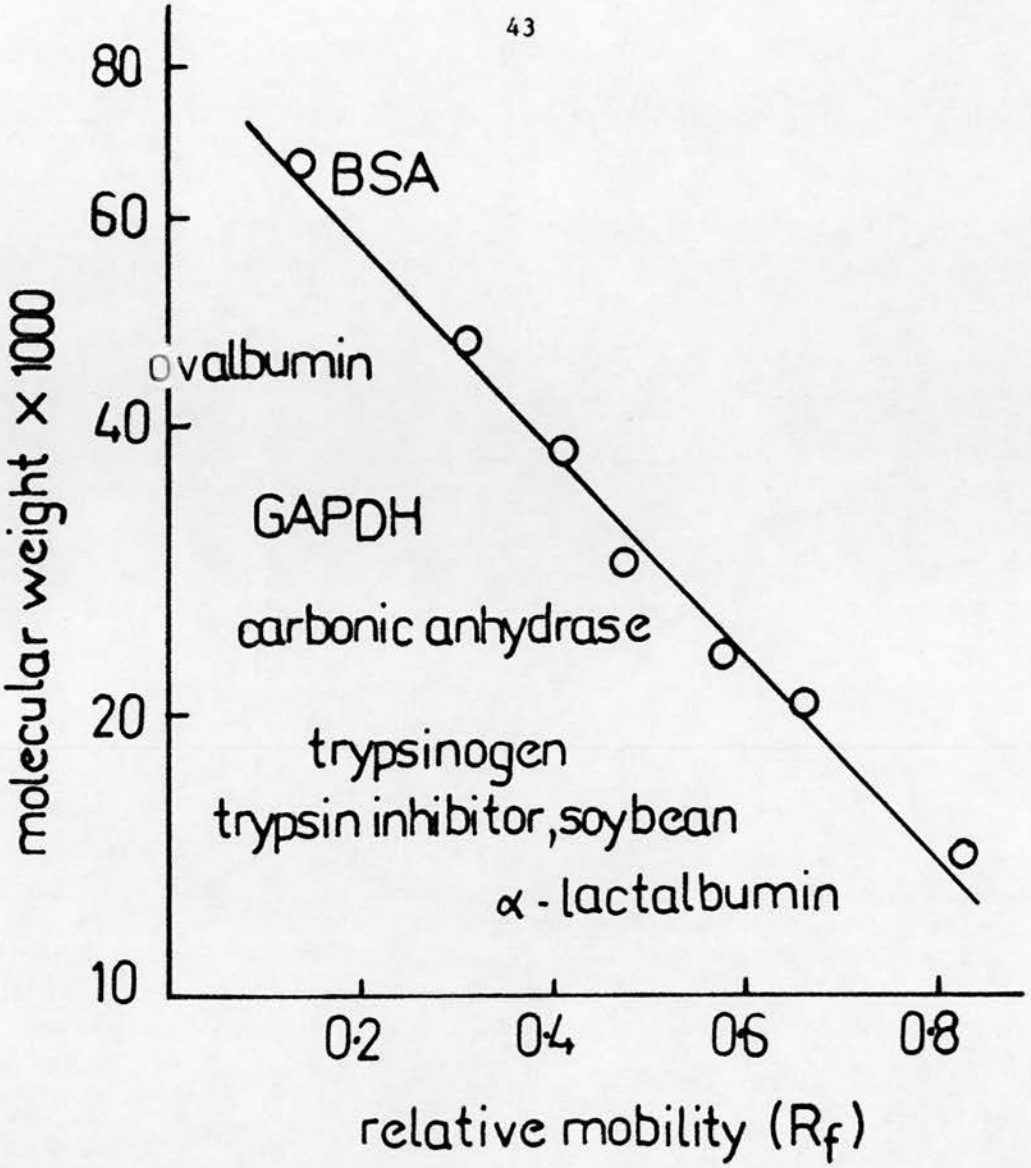


fig. 2-4 Electrophoretic mobility versus log molecular weight on 10% SDS-PAGE

The calibration curve was obtained using proteins from the Dalton Mark VII-L™ molecular weight marker kit (supplied by Sigma Chemical Co.)

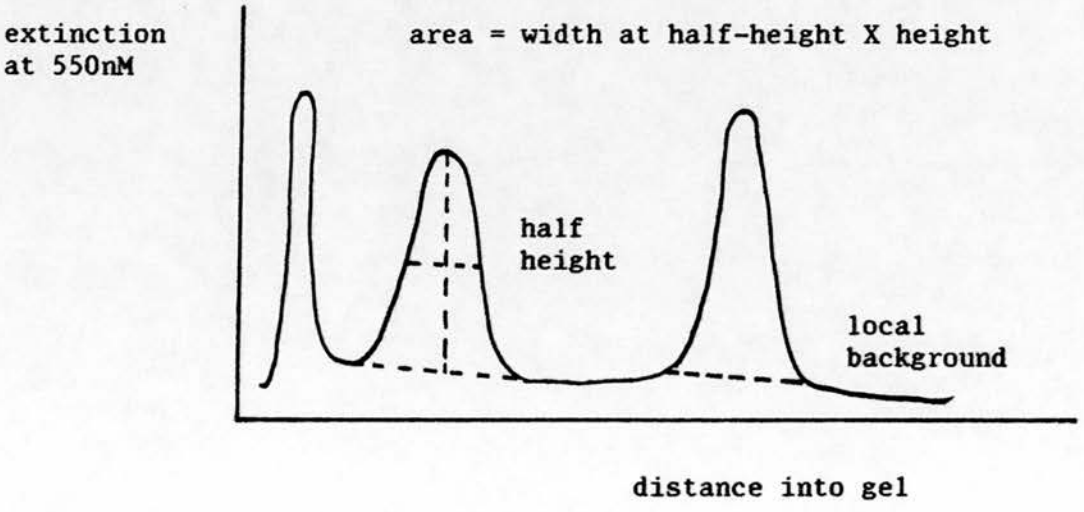
2.7.2 Autoradiography

Exposure times for films exposed to low energy β emitters like tritium can be weeks or even months. To reduce the time required for exposure gels were treated with an enhancer (New England Nuclear EN^{3}HANCE) which contains a fluor. As decay particles from the gel hit the enhancer, it starts to fluoresce, producing many photons in response to each decay particle. This enhancement significantly reduces exposure times.

Gels containing tritium-labelled protein were stained as described in section 2.7.1 except that the temperature was kept below 45°C, then immersed in the fluorographic enhancer for 30 min. with shaking. Gels were dried using a Hoeffer slab gel dryer onto Whatman 3mm paper under vacuum with heating to 60°C. The dried gels were then exposed to X-ray film at -70°C for times between seven and fourteen days and developed.

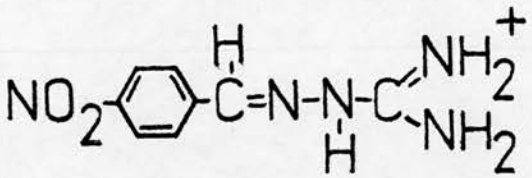
2.7.3 Densitometric analysis

Gels were scanned for E_{550} and then the amount of protein was estimated from the areas under the peaks, assuming a local background level to be the bottom of the peak.



2.8 Synthesis and assay of p-nitrobenzylidine amino guanidine (pNAG)

Guanyl hydrazones of p-nitro benzylidine and methyl glyoxal serve as acceptors of ADP-ribose groups for the reaction catalysed by cholera toxin. ADP-ribosylation lowers the pKa of pNAG by 0.7 - 0.8 pH unit* at 370nm. This change in absorbance can be used as a continuous spectrophotometric assay of cholera toxin activity (Soman *et al.*, 1983).



structure of pNAG

2.8.1 Synthesis of pNAG

The procedure followed for the synthesis of pNAG was that described by Baiocchi *et al*, (1963) for the synthesis of guanylhydrazones. Two molar aminoguanidine bicarbonate was acidified with HCl to pH5 and heated to 60°C. Two moles dry weight of p-nitrobenzaldehyde was added with constant stirring and with the addition of ethanol to keep the mixture in solution. Following continuous stirring for 16 hours the mixture was filtered and rotary evaporated. The dry product was recrystallised twice from alcohol, washed with acetone and weighed. Total yield was 21.9g which was a 38% yield from the starting weight. The product had a melting point of 247°C (lit.248°C) and absorbance maxima as described in fig. 2-5.

* causing an absorbance change

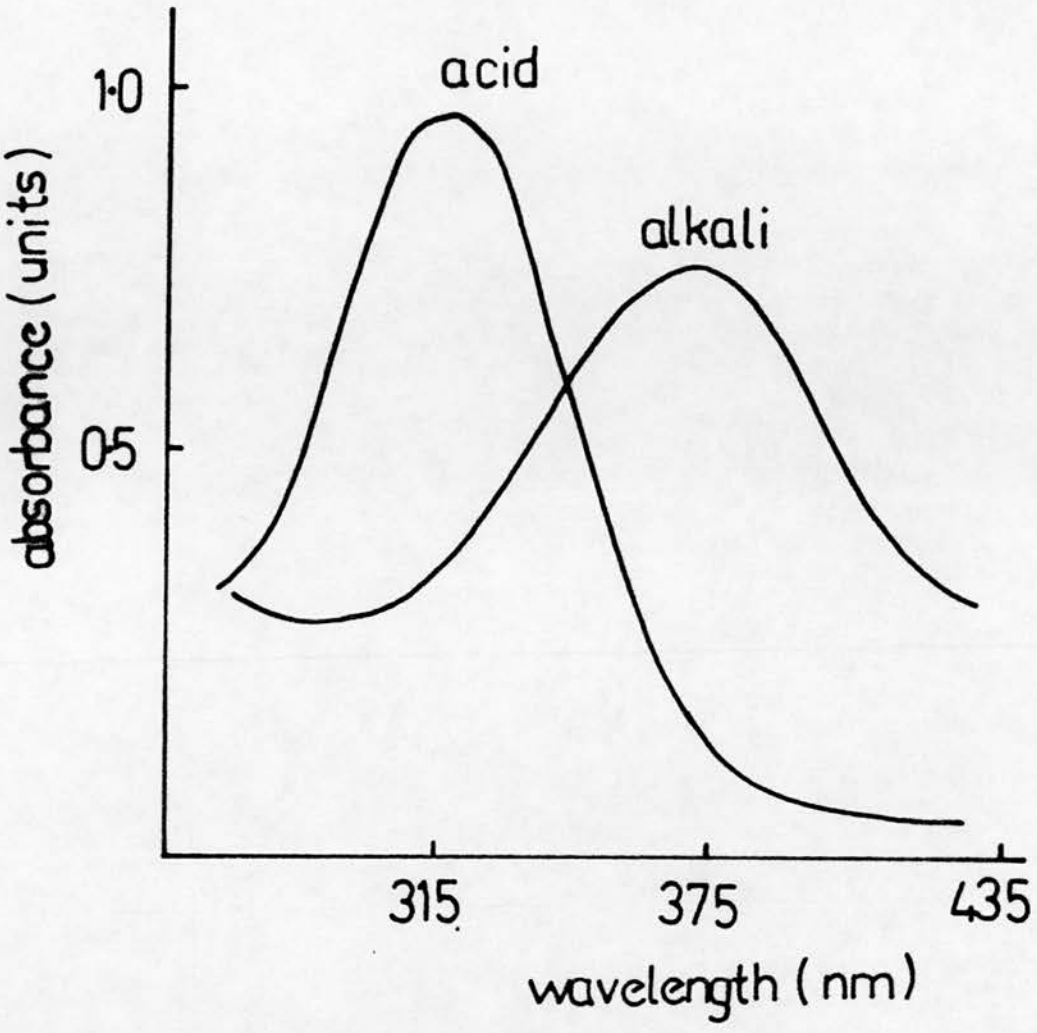


fig.2-5 Absorbance of pNAG

The absorbance of pNAG was recorded in 0.1M HCl and 0.1M NaOH. The absorbance maxima were at 315nm (ϵ = 2.1 x 10^4 mol/l/cm)and 375nm (ϵ = 1.8 x 10^4 mol/l/cm) respectively.

2.8.2 Spectrophotometric assay of ADP-ribosylation

Assays were performed using a PYE Unicam SP8-100 ultraviolet/visible wavelength spectrophotometer. Temperature equilibration at 37°C was attained by pumping liquid from a thermostated bath to the reaction chamber. The absorbance increase at 370nm was monitored continuously. Assays in a total volume of 1ml contained 0.6µM (500µg/ml) cholera toxin, 20mM tris-acetate pH7.5, 40mM DTT and 10mM NAD^+. The reaction was started by adding pNAG to a final concentration of 0.4 - 4mM. The reference cuvette contained everything except cholera toxin. After a few minutes of unsteadiness, a steady linear phase was achieved.

2.9 Assay of ADP-ribosylation

ADP-ribosylation of low molecular weight arginine derivatives was estimated indirectly by measuring the rate of [^{3}H]-nicotinamide release from [4-^{3}H]-NAD^+ (Tait and Nassau, 1984). Assays* contained 200mM potassium phosphate buffer pH6.5, 20mM DTT, various concentrations of NAD^+ containing 20nCi of [4-^{3}H]-NAD^+ (approximately 2 x 10^5 cpm), 1.2µM(100µg/ml) cholera toxin, *E.coli* heat labile toxin (LT) or cholera A_1 chain and concentrations of arginine derivatives from 0.01 - 8mM. All samples were assayed in triplicate. Incubations were conducted for up to 30 min. at 37°C and reactions terminated by dilution with 1ml water. [^{3}H]-nicotinamide was separated from the reaction mixture by ion-exchange chromatography on a 0.5 x 1cm column of QAE-Sephadex A25. Samples were loaded onto columns and washed with a further 3ml of water, the effluent being collected directly into scintillation vials. After the addition of 15ml of scintillation fluid, samples were counted

* in a total volume of 100µl

for one minute. Under these conditions NAD^+ is retained by the Sephadex columns while nicotinamide is completely eluted by the water. Columns were regenerated by washing with 5ml of 1M HCl, which removes the NAD^+, followed by 12ml of H_2O.

An alternative method of separating the nicotinamide and NAD^+ was as follows. Reactions were terminated at the appropriate time by the addition of 300µl butan-1-ol and mixing. Following centrifugation at 15000xg for 5 min., [^{3}H]-nicotinamide in the upper phase was determined by scintillation counting.

2.10 Thin layer chromatography (tlc)

The efficiency of the separation procedure in the ADP -ribosylation assay, and the breakdown of NAD^+ into nicotinamide during other procedures was routinely checked using tlc. Samples from the acid or water washes from ion exchange columns, or from the organic solvent or aqueous phase of the butanol treated samples described in 2.9 were centrifuged to remove protein. Samples of 5 - 10µl were spotted onto silica gel-coated aluminium backed plates (7.5cm x 5cm) and were developed in propan-2-ol: ethyl acetate: ammonia (9:4:3) or alternative solvents as indicated. Solutions of NAD^+ and of nicotinamide (100mg/ml) were used as markers. Plates were dried, visualised under short wave ultraviolet light and the spots scraped and counted (fig. 2-6). Typically, more than 98% of the nicotinamide and 2% of the NAD^+ were extracted into the organic phase in the butanol separation procedure, about 96% of the nicotinamide and negligible NAD^+ being eluted by water from the ion exchange columns.

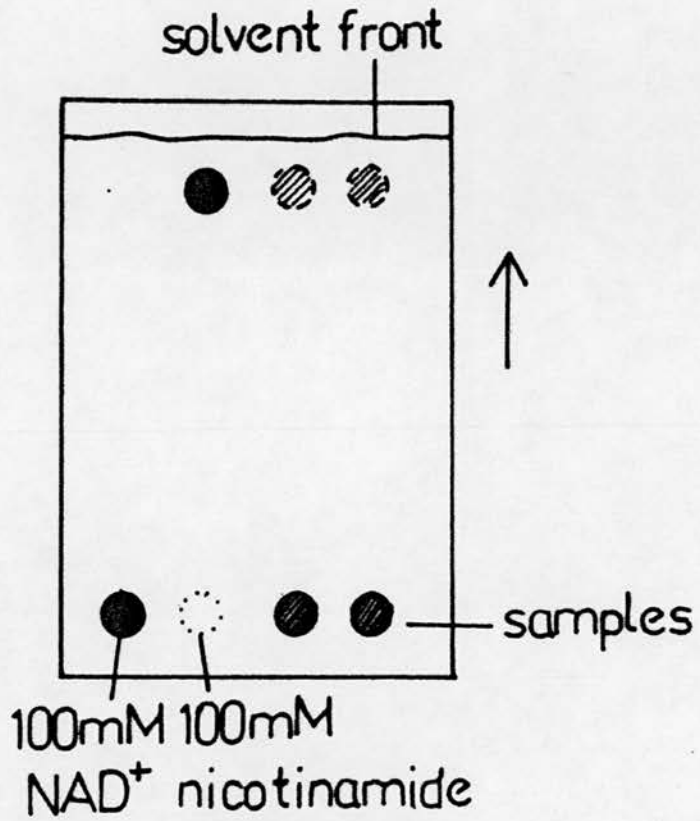
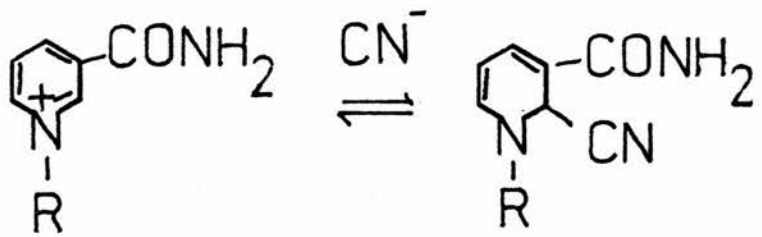


fig. 2-6 Separation of NAD^+ and nicotinamide by tlc

Samples and solvents were as described in the text

2.11 Spectophotometric detection of NAD^+ with Cyanide

The method used was derived from that of Colowick *et al.*, (1951) who first developed an assay for NAD^+ from the observation that NAD^+ reacts with cyanide to form complexes having absorption spectra resembling those obtained by enzymatic reduction of NAD^+.



Samples of between 25 and 100µl containing approximately 25 - 100µM NAD^+ or unknown derivative were diluted with either 0.9ml H_2O or 0.9ml 225mM KCN in H_2O, and the final volume made up to 1ml with H_2O. After mixing by inversion the samples were transferred to a quartz 1ml cuvette and the absorbance recorded between wavelenghts 240 - 400nm using a Carey Model 219 spectrophotometer. The reference cuvette contained the same reagents as the sample cell but with water added in place of the nucleotide solution. The absorbance coefficient at 327nm of the NAD^+-cyanide complex was taken to be 5.9 x $10^{-3}M^{-1}cm^{-1}$.

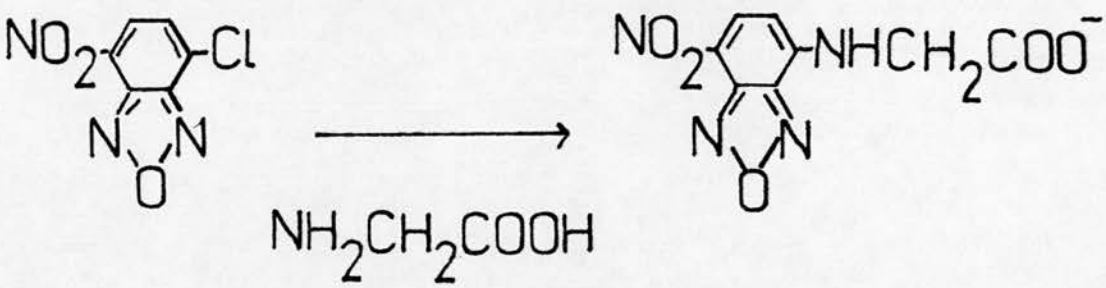
2.12 Equilibrium Dialysis

These studies were carried out in Teflon cells (Dianorm "R" Fisons Ltd.). Each cell consisted of two Teflon half-cells (volume 0.25ml, membrane surface area 2.0cm^2) separated by a membrane (Spectrapor) of molecular weight cut-off 6000 - 8000. Five such cells were arranged in an assembly that rotated gently (10 turns/ min.) in a water bath at 37°C. For binding experiments, 200mM potassium phosphate pH6.5, or buffer as indicated, containing 20mM, [4^{3}H] NAD^+ (20nCi) and concentrations of unlabelled NAD^+ from 0.1 - 100mM in a total volume of 200µl was injected into one half-cell and the same volume of buffer containing 10 - 80µM cholera toxin or its A_1 chain injected into the other half-cell. During dialysis samples were taken through ports from both sides of the membrane and the radioactivity was determined. At the end of each experiment (up to 180 min.) samples were taken from both sides of the dialysis membrane and the breakdown of NAD^+ into nicotinamide was noted using

the tlc method described in section 2.10. Typically, breakdown was negligible. The activity of dialysed protein preparations was assayed using the ADP-ribosylation assay in section 2.9.

2.13 Fluorescent labelling of cholera toxin with 4-chloro-7-nitro benzofuran (Nbf)

The fluorescent molecule 4-chloro-7-nitro benzofuran (Nbf, also known as NBD) has been shown to be a useful probe of protein structure (Ghosh and Whitehouse, 1968) (Birkett *et al.*, 1971). Nbf derivatives become highly fluorescent at low dilutions when their amino group is derived from an aliphatic amine e.g. an amino acid.



A solution of cholera toxin or *E.coli* heat labile toxin (5mg/ml) was dialysed at 4°C against 50mM triethanolamineHCl, 1mM EDTA pH8.5. A 3.9mM solution of Nbf-Cl in H_2O was prepared by stirring the Nbf-Cl in H_2O for six h. at 25°C then filtering. Its concentration was estimated from the absorbance at 345nm. Equal volumes of the toxin and Nbf-Cl solutions were mixed and incubated for 48 hours at 4°C. The solution was then exhaustively dialysed against TEAN buffer pH7.5 until no more free Nbf-Cl was detected in the dialysate. The protein concentration of the product was estimated from the absorbance at 280nm (assuming an absorption of 11.4 for a 10mg/ml

solution of toxin, (LoSpalluto and Finkelstein, 1972)) and the concentration of the amino acid-Nbf derivative was estimated from the absorption at 480nm (assuming an absorption coefficient of $26000M^{-1}cm^{-1}$, (Aboderin *et al.*, 1973)).

2.14 Fluorescent measurements

Titrations were carried out in a Perkin-elmer MPF-3000 spectrofluorometer containing a thermostatically controlled cuvette holder. The excitation and emission slits were set at 15nm and 5nm respectively. Titrations were performed by adding 5 - 10µl increments of NAD^+ in TEAN buffer pH7.5 to a 1ml solution of cholera toxin, A_1 chain or Nbf-labelled cholera toxin or subunits in TEAN buffer. Samples were stirred after each addition and the decline in protein fluorescence was measured at 333nm (excitation 285nm) or at 533nm (excitation 468nm) respectively. Samples were kept at 37°C and shielded from incident light when readings were not being taken. The fluorescence of samples was compared with that of either a solution of tryptophan or of Nbf-myoglobin of comparable initial fluorescence intensity to the sample. To correct for attenuation of the excitation beam by added ligand, the control solutions were similarly titrated and appropriate corrections were made. All data were corrected for dilution.

2.15 Photochemical labelling of cholera toxin with NAD^+

This section of work was performed in the Department of Microbial Biochemistry, Glaxo Group Research Ltd., Greenford, Middlesex and was financed entirely by Glaxo Group Research Ltd. The technique used is derived from that of Carroll and Collier (1984).

Samples of cholera toxin, 50 - 75µM (5 - 6.5mg/ml) in TEAN buffer

pH7.5 containing 20mM DTT and 5mM NAD^+ with either [nic-4^{3}H]NAD^+ or [adenine-2,8^{-3}H]NAD^+ were allowed to stand for 10 min. at 4°C. The samples were then placed as droplets of 100 - 150µl on a parafilm-coated microtitre plate (Nunc) floating in an ice-bath. The droplets were irradiated at a distance of 5cm using a CAMAG 8w dual wavelength lamp set at 254nm. At appropriate time intervals 20µl aliquots were removed and diluted into 6M urea containing 2.5% BSA. This was designed to dissociate any non-covalently bound NAD^+ from the toxin. Protein was then precipitated by adding 300µl 12% trichloroacetic acid (TCA) in 0.1M phosphoric acid and the pellet spun down, washed three times in 12% TCA and finally dissolved in 600µl H_2O. Emulsifying scintillant (10ml) was added and the samples counted for one minute.

2.16 Digestion of the A_1 chain by Trypsin in the presence of NAD^+

When cholera toxin A_1 chain was digested by trypsin in the presence of saturating concentrations of NAD^+, it was found that the NAD^+ could protect the A_1 chain from digestion. This observation was exploited in the calculation of the dissociation constant, K_d, for the binding of NAD^+ to cholera toxin as described in section 5.2.5.

Samples containing 50µM A_1 chain in TEAN buffer pH7.5 and various concentrations of NAD^+ were allowed to stand for 10 min. at room temperature. Trypsin was added to a final concentration of 5µg/ml and digestion allowed to proceed at 37°C for the appropriae time before being stopped by the addition of 1mM PMSF in propan-1-ol. Control samples contained ovalbumin instead of the A_1 chain. The digested peptide was run on 15% SDS-PAGE and stained

with coomassie blue as described in section 2.7. A Gilford densitometric scanner was used to measure the appearance of the largest digestion band of molecular weight 20000 by its absorbance at 550nm.

To determine whether or not NAD^+ had any direct effect on trypsin activity, a pH stat meter was used which records the amount of titrant used to maintain a pre-selected pH value as a function of time. This is then correlated to the activity of trypsin via the appearance of H^+ ions which are produced during the reaction. Each sample contained 250µM N-benzyl-α arginine ethyl ester (BAEE), a synthetic substrate of trypsin, in a total volume of 5ml 1mM sodium phosphate pH7.0 in the presence or absence of 40mM NAD^+. After an initial equilibration at 30°C the reaction was started by the addition of 20µl 1mM HCl containing 150µg trypsin. The reaction rate was identical in the presence and absence of NAD^+.

2.17 Amino acid analysis and sequencing

Hydrolysis was performed in evacuated tubes with 0.5ml 5.7M HCl at 110°C for 20h. Amino acid analysis was performed using one of two methods.

Quantitative analysis was carried out with a Waters amino acid analysis hplc system incorporating a Waters ion-exchange column and fluorescent detection of amino acids after reaction with hypochlorite (to convert proline to a primary amine) and o-phthalaldehyde. This analysis was kindly performed by Mr. Bryan Dunbar, University of Aberdeen, Department of Biochemistry.

Alternatively, amino acid analysis was performed with an LKB

4400 analyser in the Department of Biochemistry, University of Edinburgh with the help of Mr. Andy Cronshaw using the ion exchange method of Moore, Spackman and Stein (1958). Conditions were as outlined in the Analysis Data sheet shown in fig. 2-7.

Amino acid sequencing was done automatically with an Applied Biosystems gas phase sequencer by Bryan Dunbar. The sequencer was operated with a double cleavage, double extraction programme without a vacuum as recommended by the manufacturers. The phenyl thio hydantoin derivatives were identified by hplc on Apex Cyano columns (0.4 x 25cm) with an acetate buffer pH.5.0/acetonitrile gradient system and detection at 254nm.

2.18 Dansyl chloride end group analysis

The method used was derived from that of Gray (1967). A solution of peptide (10nMol) in 50µl was transferred to a small glass test tube with a fine-tipped micro-pipette. Ethanol (450µl) was added and the sample allowed to stand for two hours at room temperature.

ANALYSIS DATA SHEET

LKB BIOCHROM

AMPLE .....

OLUME LOADED ..... AMOUNT LOADED .....

ATE ANALYSED .....

UN NUMBER .....

PERATOR .....

	Fluor	440nm	570nm
e Line Pot Setting			
sorbance/Gain			

uffer p.H.

= Sodium Citrate pH=3.20
= " pH=4.25
= " pH=3.0
=
= Sodium Borate pH=10.0
= NaOH 0.4M

MINS.			BUFFER A	B	C	D	E	F	PUMP	T2	T3		NIN O/R	FAN	LOAD	SOR	REC				X 10	HOLD
0	0	1	1	●	o	o	o	o	o	●	o	o	o	o	o	o	●	●	o	o	o	●	o
0	4	2	2	●	o	o	o	o	o	●	o	o	o	o	o	o	●	●	o	o	o	o	o
0	7	3	3	●	o	o	o	o	o	●	●	o	o	o	o	o	●	●	o	o	o	o	o
2	1	0	4	o	●	o	o	o	o	●	●	o	o	o	o	o	●	●	o	o	o	o	o
0	2	1	5	o	●	o	o	o	o	●	o	●	o	o	o	o	●	●	o	o	o	o	o
2	1	0	6	o	o	o	o	●	o	●	o	●	o	o	o	o	●	●	o	o	o	o	o
0	2	8	7	o	o	o	o	o	●	●	o	●	o	o	o	o	●	●	o	o	o	o	o
0	7	0	8	●	o	o	o	o	o	●	o	●	o	o	o	o	●	●	o	o	o	o	o
0	4	2	9	●	o	o	o	o	o	●	o	o	o	●	●	o	o	o	o	o	o	o	o
1	3	3	10	●	o	o	o	o	o	●	o	o	o	●	o	o	o	o	o	o	o	o	o
0	0	5	11	●	o	o	o	o	o	●	o	o	o	●	o	o	o	o	o	o	o	o	o
0	1	0	12	●	o	o	o	o	o	●	o	o	o	●	o	●	o	o	o	o	o	o	o
			13	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
			14	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
			15	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

nperature setting

= 50 °C
= 58 °C
= 82 °C

Operating Pressures at T1

Buffer = 25.35 Bar
Nin/OPA = 40 Bar
Coil = 7-8 Bar

Flow Rates

Buffer = 28 ml/h
Nin/OPA = 20 ml/h
Chart Speed = 300 mm/h

Pump Settings

Buffer Pump 24.0
Nin/OPA Pump 16.5

Analytical Column

Column Length 202 mm
Column i.d. 4.6 mm
Resin Type 8µ

fig. 2-7 Analyis Data sheet for LKB analyser showing details of the citrate buffer programme

The precipitate was spun down, redissolved in 10µl sodium bicarbonate (1mM) and dried down under vacuum with 1M H_2SO_4 present to absorb ammonia. The sample was redissolved in 10µl H_2O and to this was added 10µl dansyl chloride, 2.5mg/ml in acetone. Addition of the acetone causes the pH to rise to pH 8.5 - 9.8. The final concentration of dansyl chloride was 5mM. The tubes were incubated for two hours at 37°C. During this time most of the excess reagent is hydrolysed to the sulphonic acid and the solution, previously yellow, becomes colourless. Any dansyl chloride remaining is subsequently broken down during acid hydrolysis.

When the reaction was complete the solutions were dried under vacuum. Hydrolysis was performed in evacuated tubes with 50µl 6M HCl at 110°C for 20h. When hydrolysis was complete the tubes were opened and HCl removed under vacuum over NaOH pellets.

Thin layer chromatography using polyamide plates (BDH) 7.5cm x 7.5cm was used to analyse the hydrolysed samples. Standard solutions contained approximately 10nM each of the dansyl derivatives of alanine, valine, isoleucine, proline, phenylalinine, threonine, tyrosine, aspartate, lysine and arginine. Sample or standard (2µl) was spotted onto the bottom right-hand corner of each plate 1cm from each edge, and the top left corner marked for identification. Plates were developed in one direction in 3% formic acid and then at 90° to this first in toluene: acetic acid (9:1) and then in n-butyl acetate: methanol: acetic acid (20:1:1). Plates, when dry were visualised under ultraviolet light at 365nm. Using this method, as little as 10^{-5}µmol fluorescent dansyl derivative can be detected although 10^{-4}µM per spot is the practical lower

limit. The results for a set of standard dansyl-amino acids is shown in fig. 2-8.

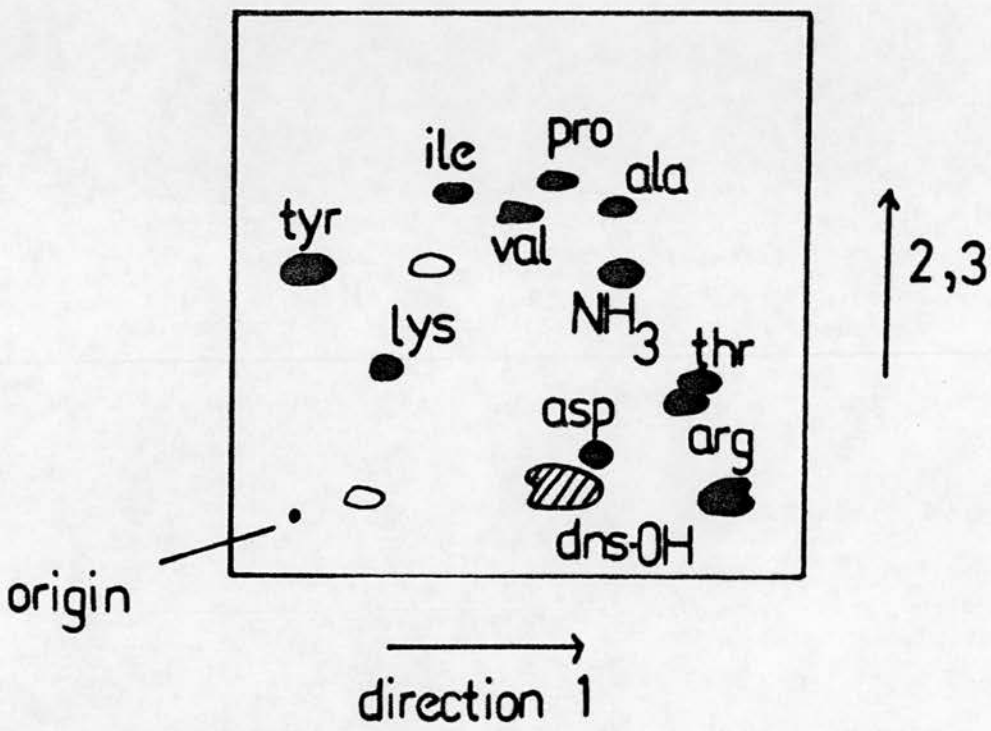


fig. 2-8 Dansyl amino acids separated by tlc

Conditions were as described in the text. Typically, sample and standards were run on opposite faces of the same sheet to allow a more accurate identification of any dansyl-amino acid in the sample.

2.19 Determination of protein

Unless otherwise stated, protein was measured using the technique of Lowry *et al.*, (1951).

2.20 Liquid scintillation counting techniques

All radioactive samples were counted in "Optiphase Safe" emulsifying scintillant from LKB. All samples were identical with respect to volume of scintillant, volume of sample, etc., and for each experiment background and standard vials were also counted. The background vial was identical to all other samples except that it contained no radioactive material. The standard vial consisted of a known amount of assay medium containing radioactive substrate. Counts arising from this vial served as an estimate of the cpm of radioactive substrate added to each assay.

Chapter 3 Kinetic analysis of cholera toxin

3.1 Introduction

Cassell and Pfeuffer (1978) first demonstrated that activation of adenylate cyclase by cholera toxin resulted from ADP-ribosylation of N_s, a regulatory subunit of adenylate cyclase. This modification leaves N_s incapable of hydrolysing its bound GTP and so induces the permanent activation of the catalytic subunit of the cyclase (Cassel and Selinger, 1977). Why ADP-ribosylation should have such an effect and why a bacterial toxin should be causing it is unclear unless the toxin is subverting a cellular regulatory mechanism. Although a number of eukaryotic ADP-ribosyl transferases have been identified (see fig. 1-6), a distinct function for mono ADP-ribosylation in the cell has not been proven.

To understand more about the catalytic activity of cholera toxin, a more detailed understanding of the kinetic mechanism would be helpful. The kinetic parameters K_m (Michaelis constant) and K_{cat}

$$\left[K_{cat} = \frac{V_{max}}{[E]}, \text{ the maximum catalytic activity when substrate is saturating} \right]$$

give an estimate of the kinetics of that reaction under the conditions likely to be found *in vivo*. Also, kinetic mechanisms and parameters can be compared between enzymes. Cholera toxin could be compared with other ADP-ribosylating toxins such as diphtheria toxin, exotoxin A and pertussis toxin, the kinetic mechanisms of which are known at least in part (Chung and Collier, 1977a; Ui, 1984; Moss *et al.*, 1983). It would be especially interesting to compare cholera toxin with eukaryotic mono (ADP-ribosyl) transferases such as the turkey erythrocyte transferase reported by

Moss and Vaughan (1978). Osborne *et al.*, (1985) recently presented estimates of the kinetic parameters and mechanism of action of this transferase.

The physiological substrate of cholera toxin, N_s, has been purified to apparent homogeneity (Northup *et al.*, 1980; Bokoch *et al.*, 1983, 1984; Codina *et al.*, 1983, 1984). However it makes up on average only one part in 10^5 of total cellular protein, is extremely labile and hydrophobic, and requires to be in a certain orientation in an appropriate membrane for activity (Gilman, 1984). It is also not the only protein in the cell membrane to be ADP-ribosylated by cholera toxin. Additional cofactors required for the reaction include NAD^+ (Gill, 1975; Moss *et al.*, 1976a; Gill and Meren, 1978), GTP (Moss and Vaughan, 1977; Enomoto and Gill, 1980), an unidentified membrane-bound protein of molecular weight 21000 (Kahn and Gilman, 1984) and possibly additional cytosolic factors (Enomoto and Gill, 1979). Obviously, a simpler system employing a more readily available analogue of N_s would make kinetic analysis easier.

Moss and Vaughan (1977) first demonstrated that cholera toxin catalyses the ADP-ribosylation of arginine, guanidine and other related compounds. This suggested that it might be an arginine side chain on N_s that was the site of modification by ADP-ribose. Indeed cholera toxin can catalyse ADP-ribosylation of other GTP-binding proteins including tubulin (Hawkins and Browning, 1982) and retinal transducin (van Dop *et al.*, 1984) which is ADP-ribosylated at the guanidinium group of an arginine residue. Other structurally unrelated proteins including follicle stimulating hormone, luteinising hormone and adrenal corticotrophic hormone will accept

ADP-ribose (Trepel *et al.*, 1981). At high concentrations the toxin can even ADP-ribosylate its own A_1 chain at an arginine residue (Lai *et al.*, 1983). An available arginine may be all that is required to allow certain proteins to be ADP-ribosylated although as the total percent of arginine in the acceptor is not directly proportional to its ability to incorporate ADP-ribose, other factors must be important too.

Tait and Nassau (1984) developed a model assay system for cholera toxin using low molecular weight arginine derivatives in place of N_s. The assay measures toxin activity indirectly by measuring the rate of toxin-catalysed splitting of NAD^+ into nicotinamide in the presence of an arginine derivative which acts as an acceptor for the ADP-ribose group. They examined a large number of potential substrates and found that the side chain of the arginine derivative was important in determining whether or not the compound would be ADP-ribosylated. They proposed that cholera toxin preferentially ADP-ribosylates guanidine groups when they are associated with a hydrophobic domain devoid of charge centres near the guanidine. This agreed with findings by Mekalonos *et al.*, (1979a) who reported that ^{125}I-guanyl tyramine was a more efficient substrate than guanyl tyramine alone. Presumably the halogenation of the aromatic group causes a decrease in the dipole moment of the phenolic hydroxyl resulting in increased hydrophobicity.

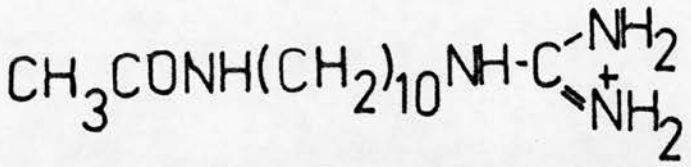
The kinetic mechanism of cholera toxin has been studied previously using arginine derivatives (Mekalonos *et al.*, 1979a; Osborne *et al.*, 1985) with differing results. Here two of the compounds characterised by Tait and Nassau (1984), [10-[(phenyl methyl) amino] decanyl] guanidine (4406) and [12-[(phenyl methyl) amino] dodecanyl] guanidine (A599) are used in a study of the kinetic parameters of cholera toxin, using the indirect assay described in the same paper. Various competitive inhibitors of NAD^+ and alternative substrates for N_a are also considered in an attempt to gain further information about the nature of the toxin's activity.

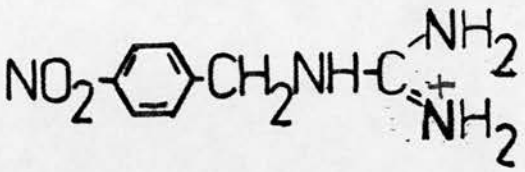
3.2 Results

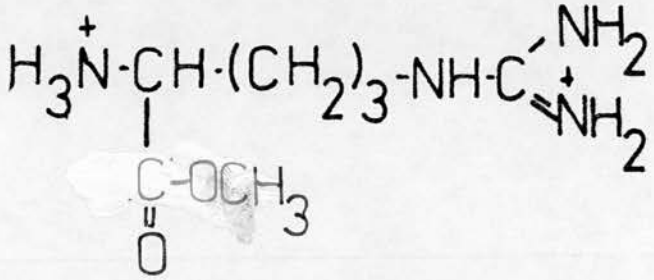
3.2.1 ADP-ribosylation catalysed by the A_1 chain

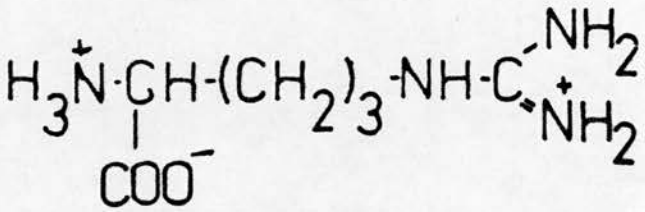
As gram quantities of the isolated A_1 chain of cholera toxin were available, preliminary experiments were performed to check its suitability for kinetic measurements. It is well established that the A_1 chain is responsible for the catalytic activity of cholera toxin, and that the catalytic activity is enhanced when the toxin is treated with high concentrations of sulphydryl-containing compounds (such as DTT) and chaotropic agents such as SDS at low concentration (Bitensky, 1975; Gill and Meren, 1978; Gill, 1975; Wheeler *et al.*, 1976). These treatments are known to promote dissociation of the A and B subunits and to reduce the disulphide link between the A_1 and A_2 chains. This suggested that isolated, reduced A_1 chain itself might be suitable for studying the enzyme activity of the toxin.

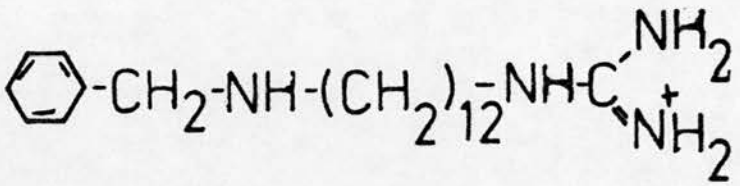
Using 2mM A599 (one of the arginine derivatives shown in fig. 3-1) as a substrate, the activity of preparations of isolated A_1 chain or of whole toxin were tested using the ADP-ribosylation assay described in section 2.9. As shown in fig. 3-2, the release of nicotinamide from NAD^+ catalysed by the A_1 chain was linear over 30 min. but when whole toxin was used there was a slight lag of about 5 min. When the whole toxin was preincubated with 20mM DTT at room temperature for 15 min., the lag was no longer seen (fig. 3-2). This suggests that the lag is presumably the time taken for activation of the toxin whereby the catalytically active A_1 chain is released by reduction of the disulphide bond that attaches it to the rest of the molecule.

1/ 

2/ 

3/ 

4/ 

5/ 

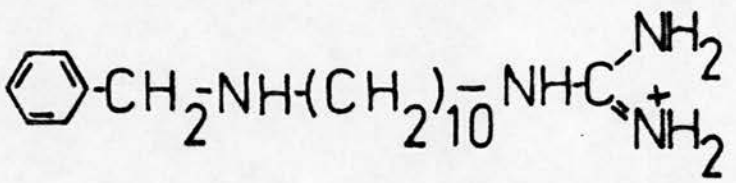
6/ 

fig. 3-1 Arginine derivatives used as substrates for ADP-ribosylation

1. [10-(acetylamino) decyl] guanidine = A603
2. p-nitrobenzylidine amino guanidine = pNAG
3. arginine methyl ester
4. arginine
5. [12-[(phenylmethyl) amino] dodecanyl] guanidine = A599
6. [10-[(phenylmethyl) amino] decanyl] guanidine = 4406

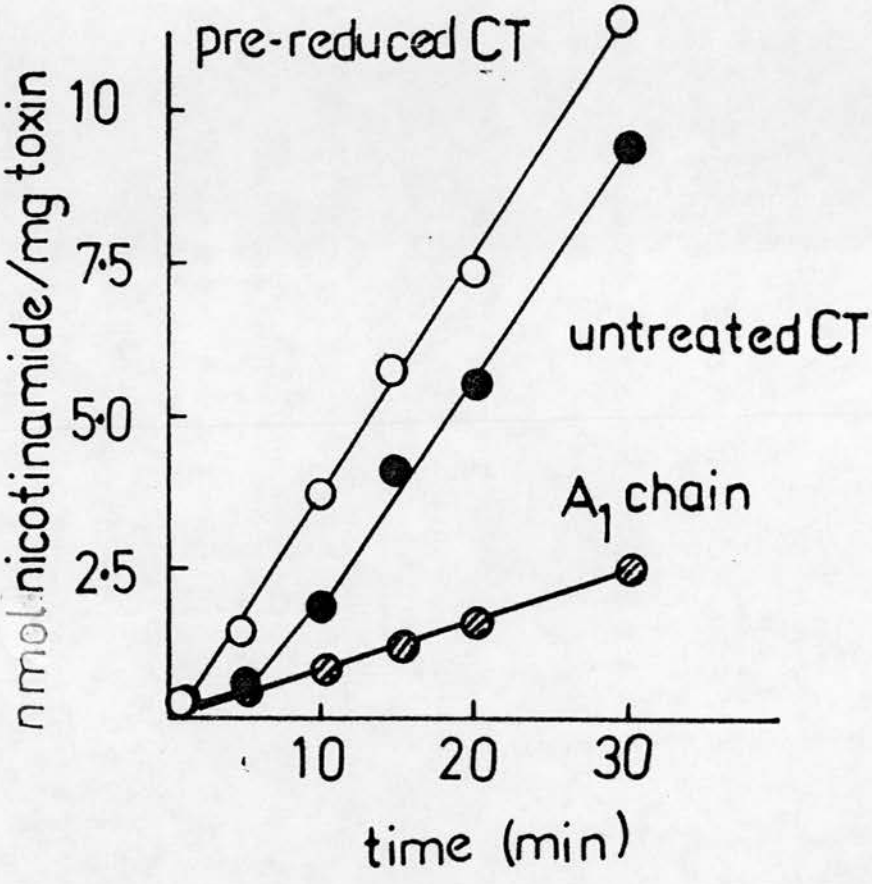


fig. 3-2 Rate of ADP-ribosylation reaction

using different toxin preparations

Cholera toxin (100µg/ml) was either untreated or preincubated with 20mM DTT for 15 min. at room temperature. The A_1 chain was 25µg/ml. Assays were as described in section 2.9 with 2mM A599 and 2mM NAD^+

As shown in fig. 3-2, the preparation of A_1 chain consistently showed much lower specific activity than did whole toxin. It seemed to lose activity on standing, even when kept frozen. Contamination with proteases was suggested by the gradual degradation of the sample over a period of weeks, (this degradation was not seen for the sample of whole cholera toxin, which came from a different supplier). At concentrations above 1mg/ml (40μM) the A_1 chain became increasingly insoluble (see section 5.2.1.4). When in solution at low concentrations it would gradually dissappear, presumably through sticking to glassware. This phenomenon has been reported by others (Gill, 1976a; van Heyningen, 1980). In attempts to increase the activity of the A_1 chain, the assay was repeated using different arginine substrates (fig. 3-1) and using buffers of differing concentration and/or pH, but no increase in the level of activity was achieved. The A_1 chain itself was dissolved and stored in TEAN buffer, pH7.5; 0.1M borate, pH8.5; 0.1M sodium phosphate, pH7.5 containing 2.5M urea, or 0.4M potassium phosphate, pH6.5, but none of these buffers increased the activity of the A_1 chain or prevented its gradual degradation, precipitation and/or disappearance.

The practice of including SDS in the preincubation of cholera toxin suggests that the toxin must have a fairly stable conformation. However, the primary sequence of the A_1 chain contains a high proportion of hydrophobic residues in its centre and secondary structure predictions suggest that it may exist mostly in a random coil configuration, a structure which might enable it to pass easily through the plasma membrane (Yamamoto *et al.*, 1984;

Duffy and Lai, 1986). This could mean that without the stabilising influences of the A_2 chain and B subunit (both of which contain much more α helix and β sheet and are therefore more rigid (Yamamoto *et al*, 1984)), and with no membrane present, the A_1 subunit cannot maintain an active conformation.

In order to test this theory, a sample of whole toxin (5mg) was separated into its constituent subunits, and portions of each subunit were reconstituted as described in Materials and Methods. The original toxin, separated subunits and reconstituted toxin were all tested for activity using the ADP-ribosylation assay with A599 as a substrate. The results, shown in table 3-1 show that while the separated A subunit had lower activity compared to the original toxin, the activity compared to the original toxin, the activity of the reconstituted toxin was higher than that of the A subunit alone, (although not as high as the original). This suggests that the A subunit, if stored on its own, does lose something of its active conformation and that at least some of its activity is restored upon reconstitution.

toxin preparation	concentration (μM)	activity (nMol. nicotinamide/mg /30min.)
whole toxin	12	0.33
A subunit	12	0.20
B subunit	15	0.04
A_1 chain	12	0.13
reconstituted toxin	12	0.23

Table 3-1 ADP-ribosylation activity catalysed by different toxin preparations

Assays contained 20mM DTT, 2mM NAD^+ with 20nCi [$4^{-3}H$] NAD^+, 2mM A599 and toxin preparations as above in a total volume of 100μl KH_2PO_4, pH6.5. Samples were incubated at 37°C for 30 min. then treated as described in section 2.9

An alternative explanation of the low activity of the A_1 chain preparation is that the protein could have lost a few residues from one end or other, affecting its active site. However Lai *et al.*, (1981) showed that 50% of the activity of the A_1 chain is retained by a fragment which lacks almost 50 residues. The loss of only a few residues would be unlikely, then, to alter the activity of the A_1 chain so drastically. To check the integrity of the sample, a portion was subjected to dansyl chloride end group analysis as described in Materials and Methods. Only one dansyl-amino acid derivative was evident, corresponding to dansyl-aspartate/ asparagine. This agrees with published primary sequence data (Duffy *et al.*, 1985) which give asparagine to be the amino terminal residue of the A_1 chain. At least this end of the A_1 chain appears to be intact.

In view of all these problems, the isolated A_1 chain was not used for kinetic analysis and whole toxin, prereduced to eliminate the lag in the time course in fig. 3-2, was used instead.

3.2.2 Characterisation of the ADP-ribosylation assay

Preliminary experiments were conducted to check the suitability of the assay for kinetic measurements. Using 2mM A599 as a substrate, enzyme activity was found to be proportional to enzyme concentration over a ten fold concentration range of 10 - 100µg/ml. The activity was also dependent on temperature and increased between 25°C and 37°C above which it started to decrease again (fig. 3-3). The reaction could be reversed by the addition of 100mM nicotinamide and a lowering of the pH to pH 6.0 (fig. 3-4). These observations are consistent with the notion that this is an enzyme-catalysed

reaction. The linear progress curve indicates that the initial conditions of the assay apply throughout the assay time, i.e. there has been no depletion of substrate, inactivation of enzyme or product inhibition.

3.2.3 Identification of a putative ADP-ribose - A599 complex

The assumption made in this assay system is that the arginine derivatives used to enhance the rate of nicotinamide release from NAD^+ by serving as substrates for ADP-ribosylation by cholera toxin. This assumption has been verified for arginine methyl ester (Moss and Vaughan, 1977). In fig. 3-5 the results are presented of an experiment to isolate the putative ADP-ribose product. A standard assay was run as described in Materials and Methods except that [4^{-3}H]-NAD^+ was replaced by [carbonyl-U^{-14}C]-NAD^+ and the products of the reaction were analysed by tlc. With the solvent used, NAD^+ and free ADP-ribose, which both carry a net negative charge remain at the origin, while the positively charged nicotinamide migrates with the solvent front. In the absence of A599 all the radioactivity remained at the origin but, with A599 present, a new peak of radioactivity appeared with Rf = 4cm. Although a detailed structural analysis of this species was not attempted, its mobility agrees with the probable charge of an ADP-ribose derivative.

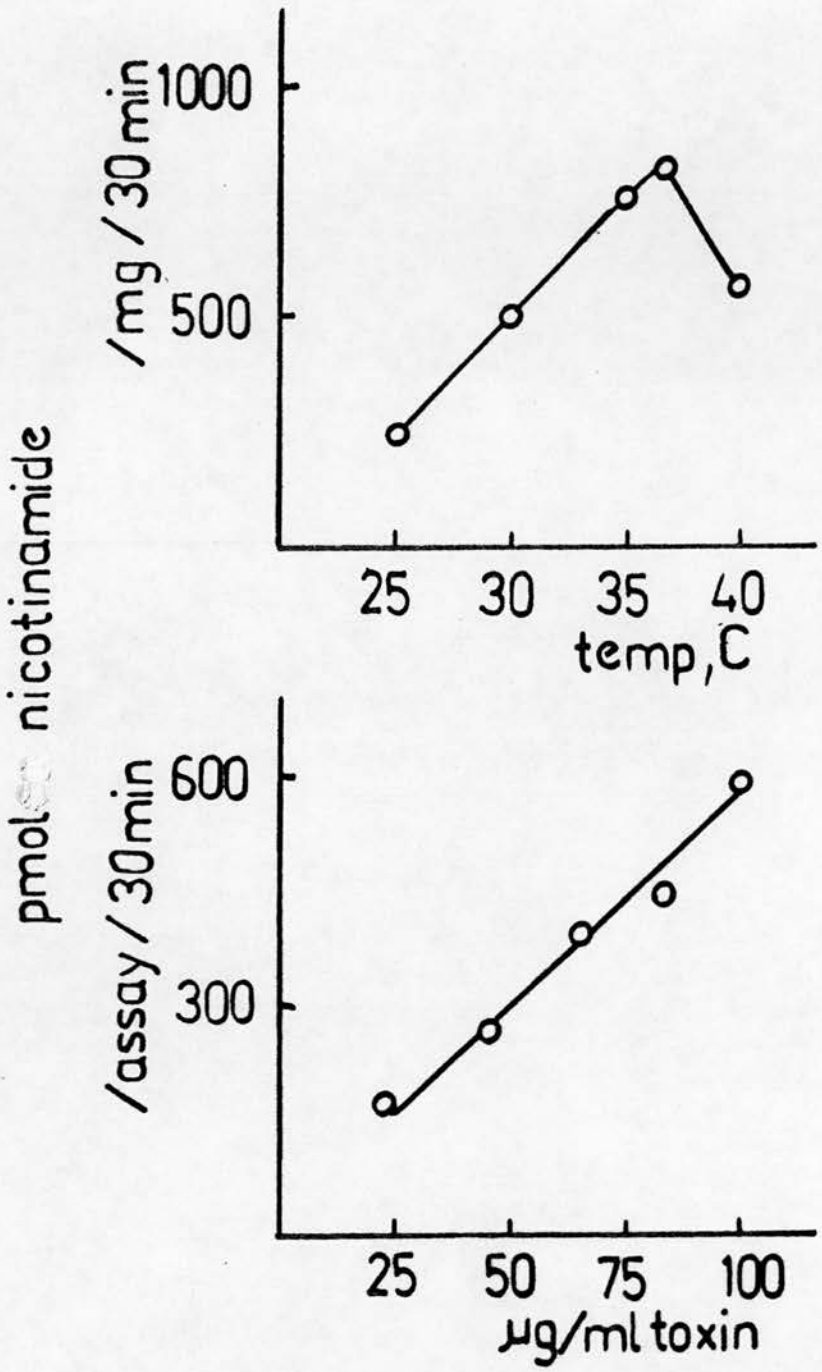


fig. 3-3 Characterisation of the ADP-ribosylation assay

The effect of temperature was studied using 2mM NAD^+, 2mM A599 and 100µg/ml cholera toxin. All other conditions were as described in section 2.9. ADP-ribosylation activity as a function of cholera toxin concentration was measured at 37°C with conditions as described in the text.

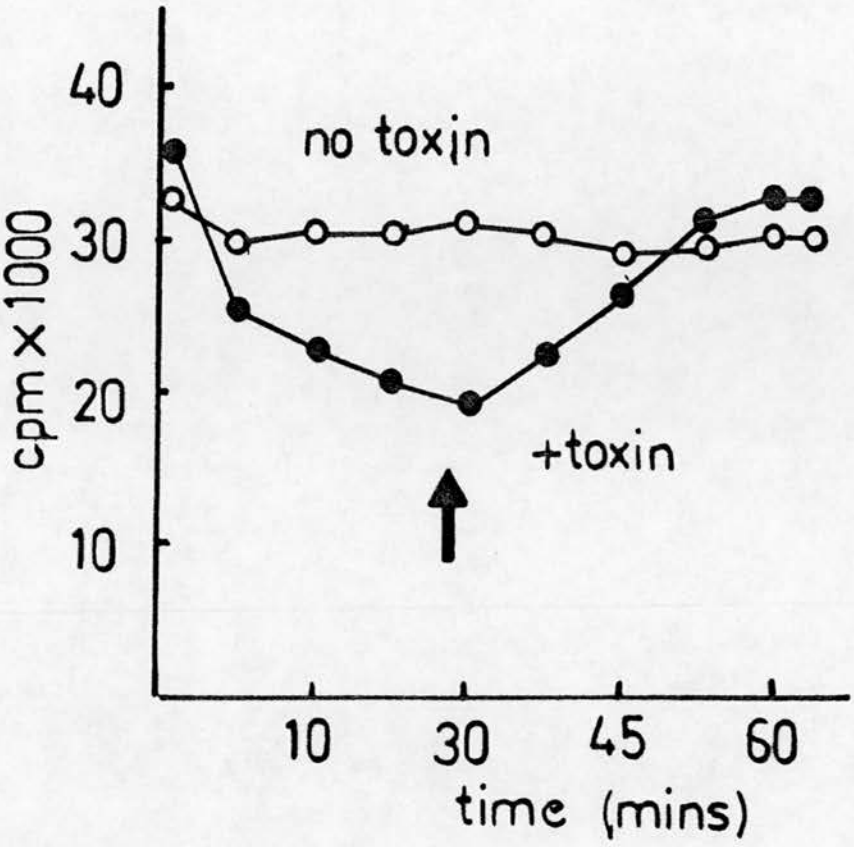


fig. 3-4 Reversal of the ADP-ribosylation assay

Assays contained 20mM DTT, 2mM NAD^+, 2mM A603, 20nCi [U-^{14}C]-NAD and 100µg/ml cholera toxin in a total volume of 200µl KH_2PO_4, pH6.5 The control sample contained buffer in place of toxin. At the times indicated, 10µl samples were removed and analysed on tlc plates as described in section 2.10. Spots corresponding to NAD^+ were cut out and counted.
The arrow marks the point where 100mM nicotinamide was added and the pH adjusted to pH6.1 with HCl

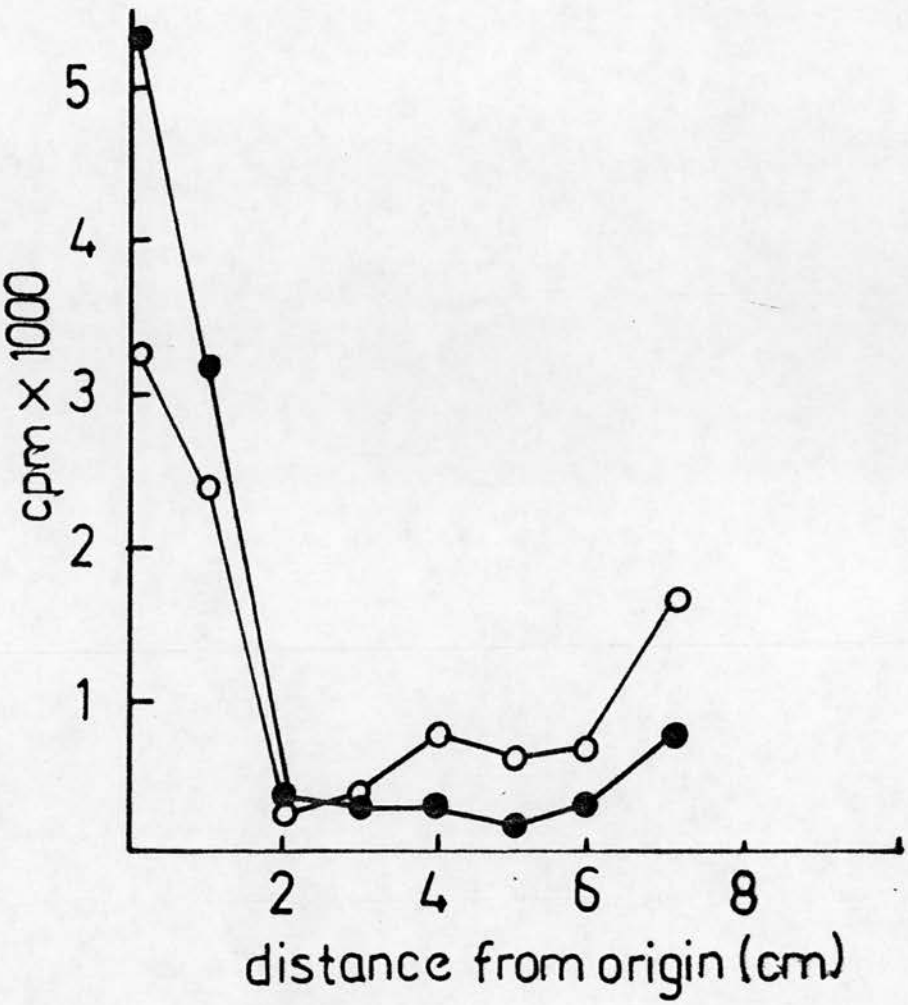


fig. 3-5 Identification of a putative ADP-ribose A599 complex

Assays in the absence (-●-) or presence (-o-) of 2mM A599 were conducted as described in Materials and Methods except that [4-^{3}H] NAD^{+} was replaced by [U-^{14}C] NAD^{+} and incubations were conducted for 60 min. Assay mixtures were immediately applied to an aluminium-backed silica coated plate (5cm x 7.5cm) and chromatographed in a sealed tank with n-butanol/acetic acid/water (2:1:1) as solvent. The plate was dried and cut into 0.5cm long segments, then counted for radioactivity

3.2.4 Controls for NAD^+ breakdown

As well as transferring ADP-ribose to N_{α}, other proteins or to arginine and its derivatives, cholera toxin can also catalyse the transfer of ADP-ribose to water, in which case the activity is called NAD^+ Glycohydrolase (or more commonly, NAD'ase) activity. This activity occurs at a low level both in the presence and absence of alternative acceptors such as A599 (Moss *et al.*, 1976b, 1983; Ueda *et al.*, 1975; Katada *et al.*, 1983). As this assay measures the release of nicotinamide catalysed by cholera toxin and not the direct formation of an ADP-ribose-acceptor, it was important to include controls to take account both of nicotinamide formed due to the toxin's NAD'ase activity, to possible contaminating NAD'ase activity and to non-enzymic breakdown of NAD^+. This latter can occur at pH values of 7.0 or above, especially in the presence of phosphate (Colowick *et al.*, 1951). However phosphate has been shown to be optimum for activity in this assay (Tait and Nassau, 1984; Moss *et al.*, 1976a). The assays were therefore performed using phosphate buffer but at a slightly acid pH of pH 6.5 to minimise degradation of NAD^+.

Controls were included for each assay sample. These contained identical concentrations of each reagent except that they had no A599. Values for these controls were typically under 5% of sample values and were a measure of NAD'ase activity. The control value was subtracted from the blank value in each case. Additional controls were included at each concentration of NAD^+ to measure non-enzymic breakdown of NAD^+. These controls contained everything except enzyme. Non-enzymic breakdown of NAD^+ was typically negligible

under the conditions of the assay for up to 30 min. at 37°C.

3.2.5 Kinetics of the NAD'ase reaction

The rate of [4-^{3}H]nicotinamide release from [4-^{3}H]-NAD^+ catalysed by cholera toxin was measured at various concentrations of NAD^+ in the absence of any artificial acceptor. This was therefore a measure of the toxin's NAD'ase activity. Results are presented as a direct linear plot of substrate concentration versus velocity (Cornish-Bowden and Eisenthal, 1978) in fig. 3-6. The K_m for NAD^+ calculated from this data was 3mM with a k_{cat} value of 0.1 sec^{-1}. The k_{cat} value assumes that all protein present is enzyme and that all the enzyme is active.

3.2.6 Kinetics of the ADP-ribosylation reaction

The initial velocity of [4-^{3}H] nicotinamide release was then measured at various concentrations of NAD^+ and of either A599 or 4406. The concentrations of each substrate were originally designed to span estimated K_m values of 4mM for the NAD^+ and 0.1mM for the A599, but had later to be extended when computer analysis of the data obtained showed both substrates to be sub-saturating. Direct linear plots of the results are shown in figs. 3-7 and 3-8. The estimated K_m values were K_m = 45 - 51mM for NAD^+ and K_m = 0.40 - 0.41mM for A599 and 440. This indicates that in the presence of the artificial acceptors, the affinity of the toxin for NAD^+ appears to have fallen. k_{cat} was estimated to be 4.9 sec^{-1}.

figs. 3-6 - 3-8 Kinetic parameters illustrated by the Direct Linear Method

In figs. 3-6, 3-7, 3-8, kinetic parameters were calculated using computer programmes described in Appendix A. The data are illustrated here using the Direct Linear Plot as described by Eisenthal and Cornish-Bowden (1974). In this method, each experimental observation of substrate concentration and velocity (s and v) is marked off on the -x or -y axis and a line is drawn through the two points and extended into the first quadrant. For a theoretically ideal experiment, all lines will intersect at a common point in the first quadrant whose co-ordinates provide values for V_{max} and K_m.

In real situations which are subject to error, lines will not intersect at a common point and in these cases V_{max} and K_m values are taken as the median of all points of intersection. When there are an even number of observations the median is taken as the mean of the middle two estimates, and where more than two lines intersect at a common point, the total number of intersections taken into account in calculating the median value is $\frac{1}{2}n(n-1)$ where n = the number of intersecting lines.

The theory behind this method is discussed in detail by Eisenthal and Cornish-Bowden (1974).

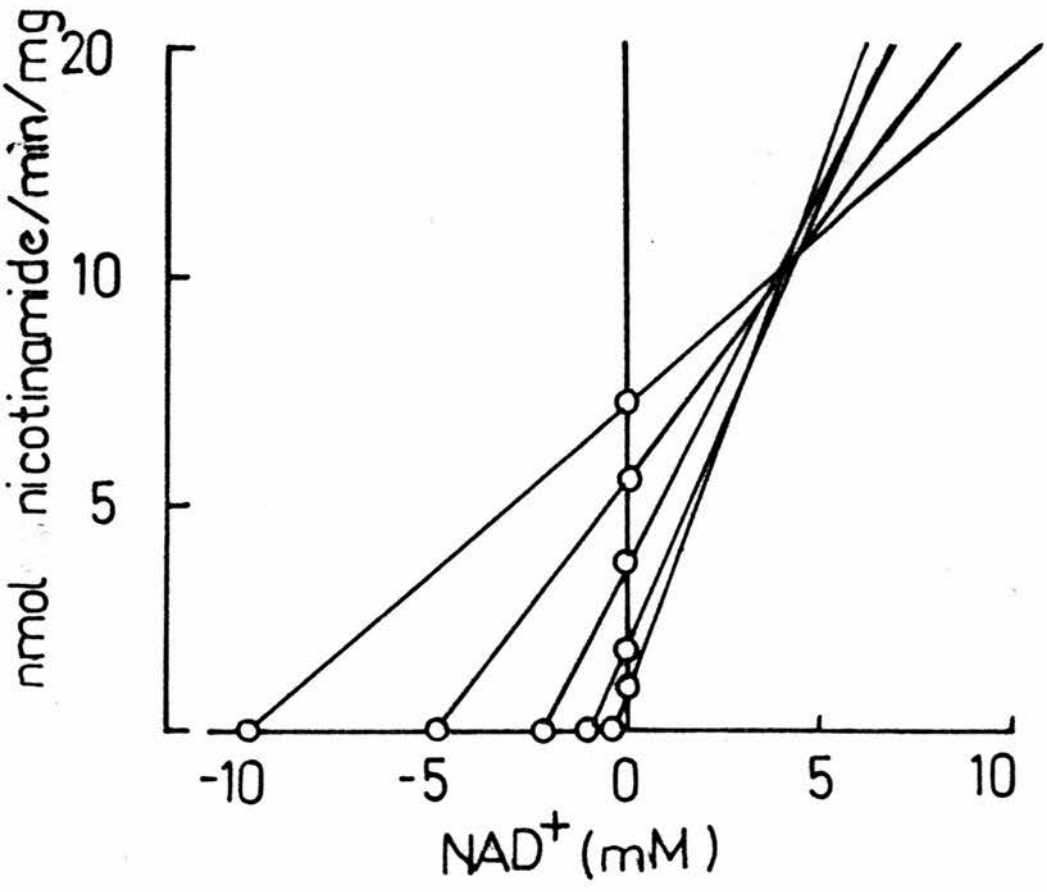


fig. 3-6 Kinetic analysis of the NAD'ase reaction catalysed by cholera toxin

Assays were conducted in a total volume of 100µl containing 200mM KH_2PO_4, 20mM DTT, 20nCi [4-^{3}H] NAD^+, 100µg/ml cholera toxin and concentrations of NAD^+ as indicated at pH6.5. Samples were treated as described in Materials and Methods. A K_m of 3mM for NAD^+ was calculated from this data.

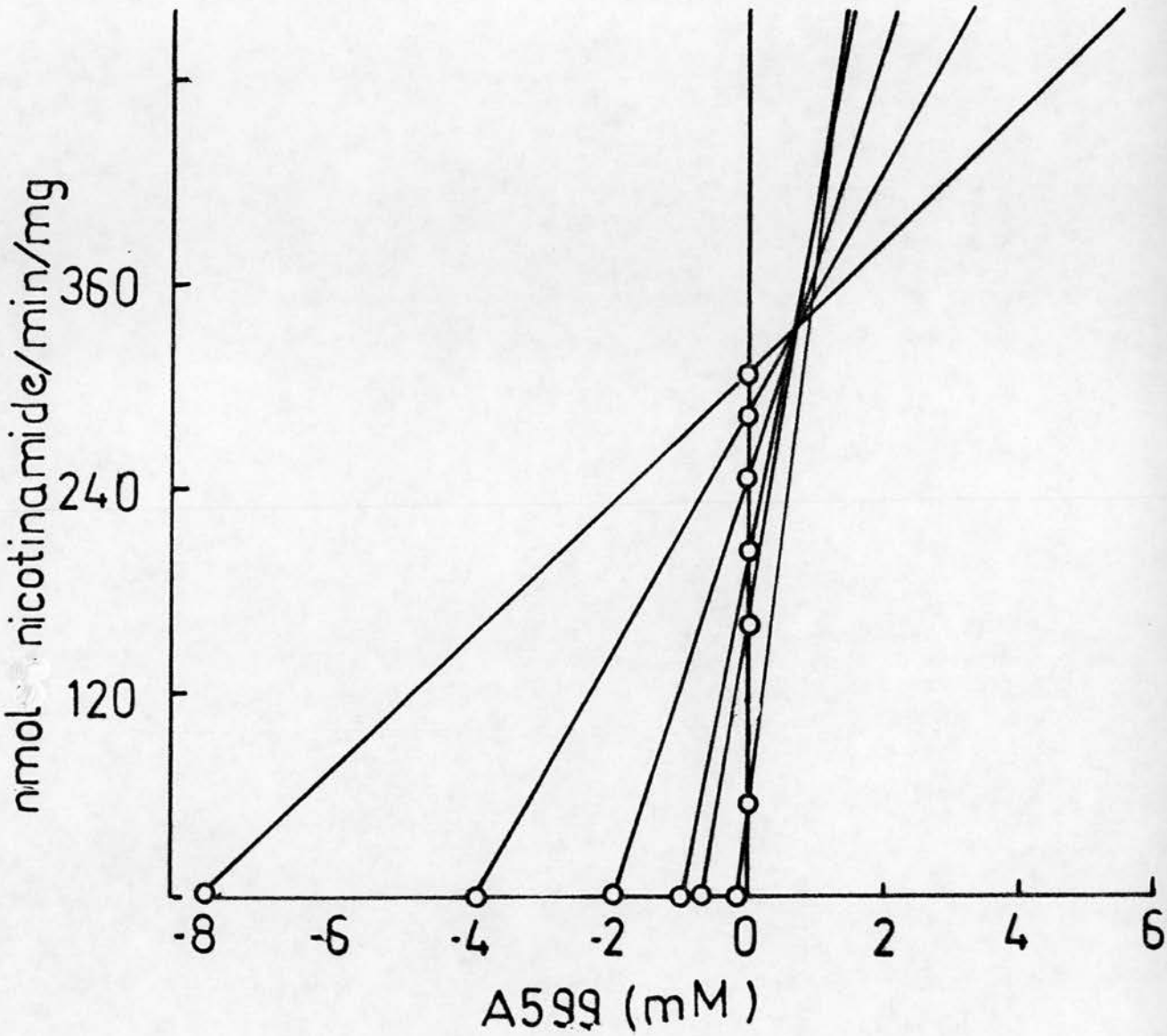


fig. 3-7(a) Kinetic Analysis of ADP-ribosylation activity catalysed by cholera toxin

Assays contained 20mM DTT, 20nCi [4-^{3}H] NAD^{+}, 5mM NAD^{+}, 100µg/ml cholera toxin and the indicated concentrations of A599 in a total volume of 100µl 200mM KH_2PO_4, pH6.5. In fig. 3-7(b), the A599 concentration was 2mM and the NAD^{+} concentrations were as indicated. Samples were treated as described in Materials and Methods. The K_m values calculated from these data were 45mM for NAD^{+} and 0.40mM for A599.

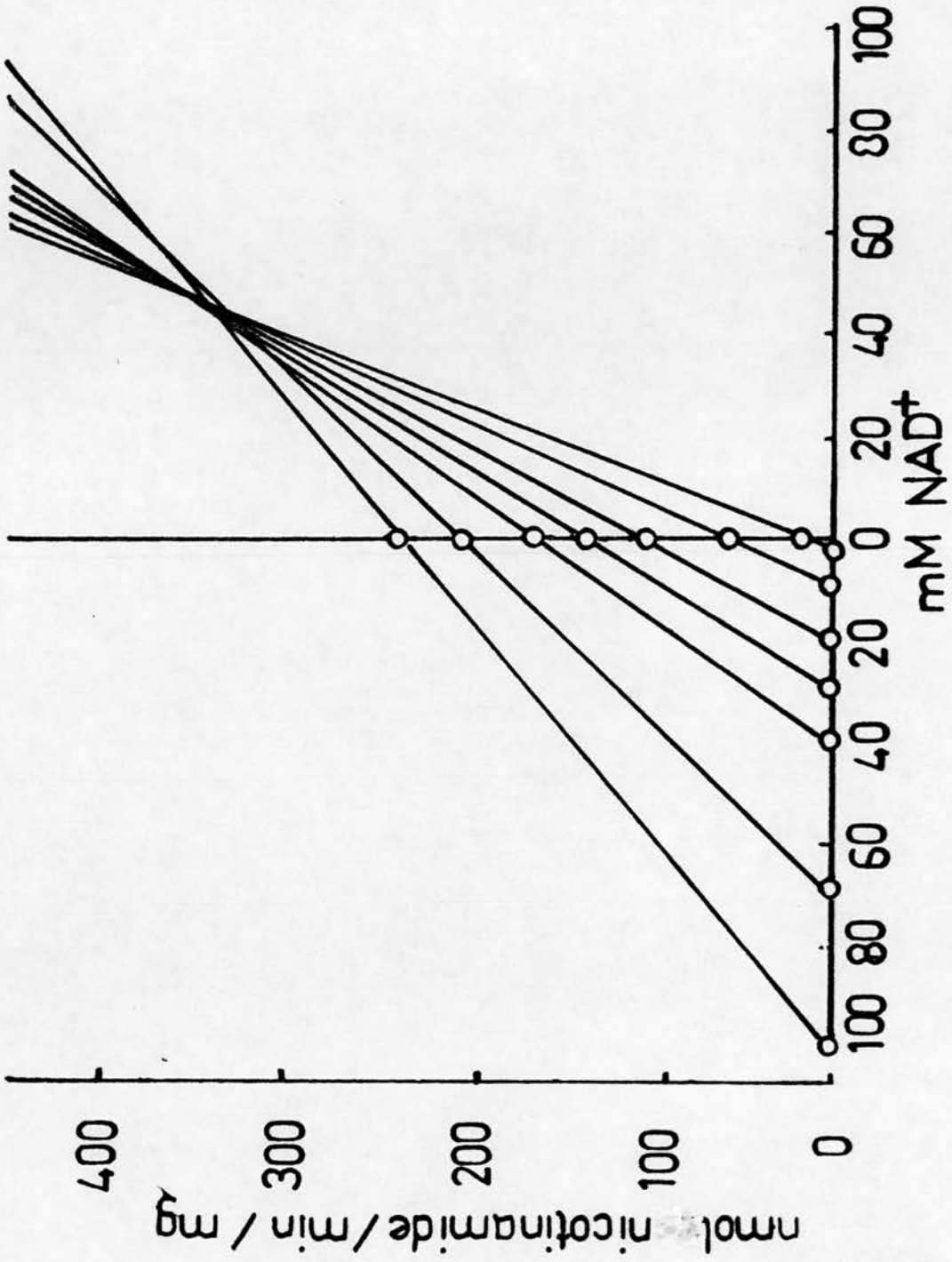

nmol nicotinamide / min / mg
0
100
200
300
400
100 80 60 40 20 0 20 40 60 80 100
mM NAD+

fig. 3-7(b)

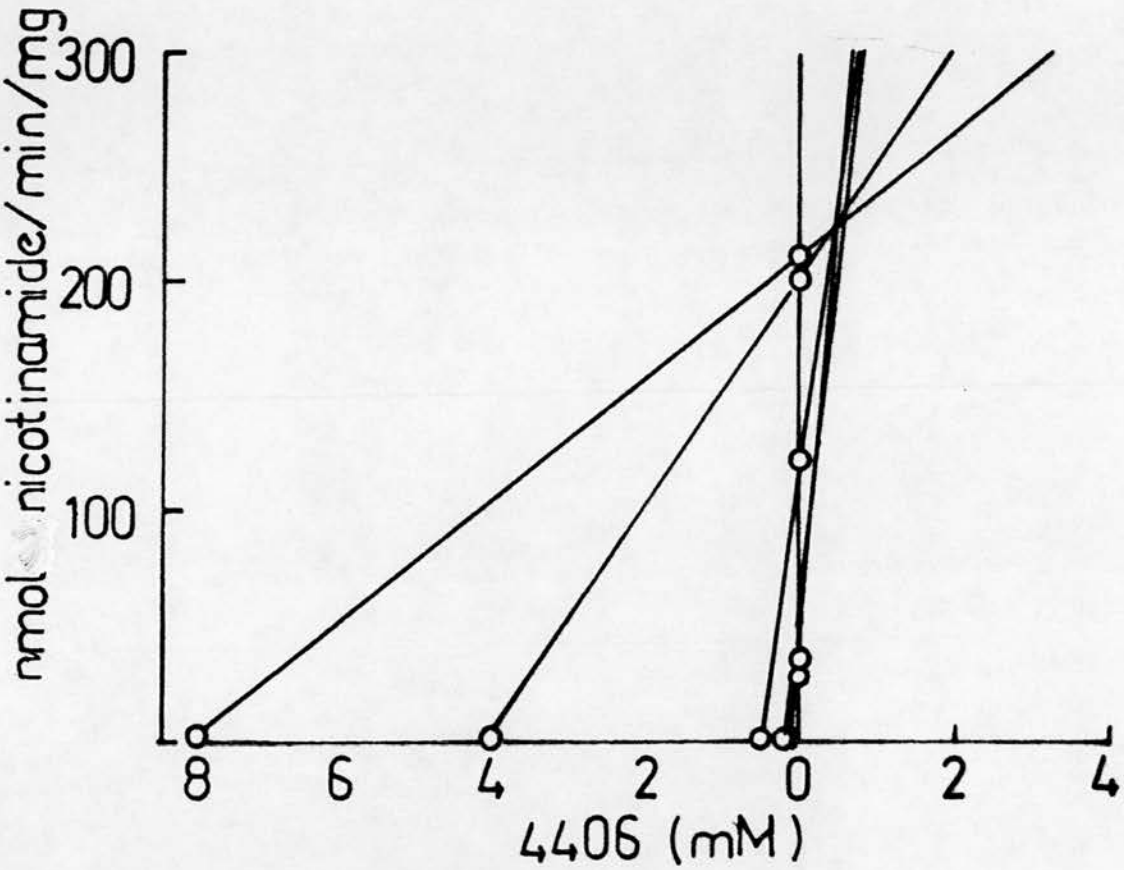


fig. 3-8(a) Kinetic analysis of ADP-ribosylation activity catalysed by cholera toxin

Assays contained 20mM DTT, 20nCi [4-^{3}H] NAD^+, 5mM NAD^+, 100µg/ml cholera toxin and the indicated concentrations of 4406 in a total volume of 100µl 200mM KH_2PO_4, pH6.5. In fig. 3-8(b) the 4406 concentration was 2mM and the NAD^+ concentrations were as indicated. Samples were treated as described in materials and methods.
The K_m values calculated from these data were 51mM for NAD^+ and 0.41mM for 4406.

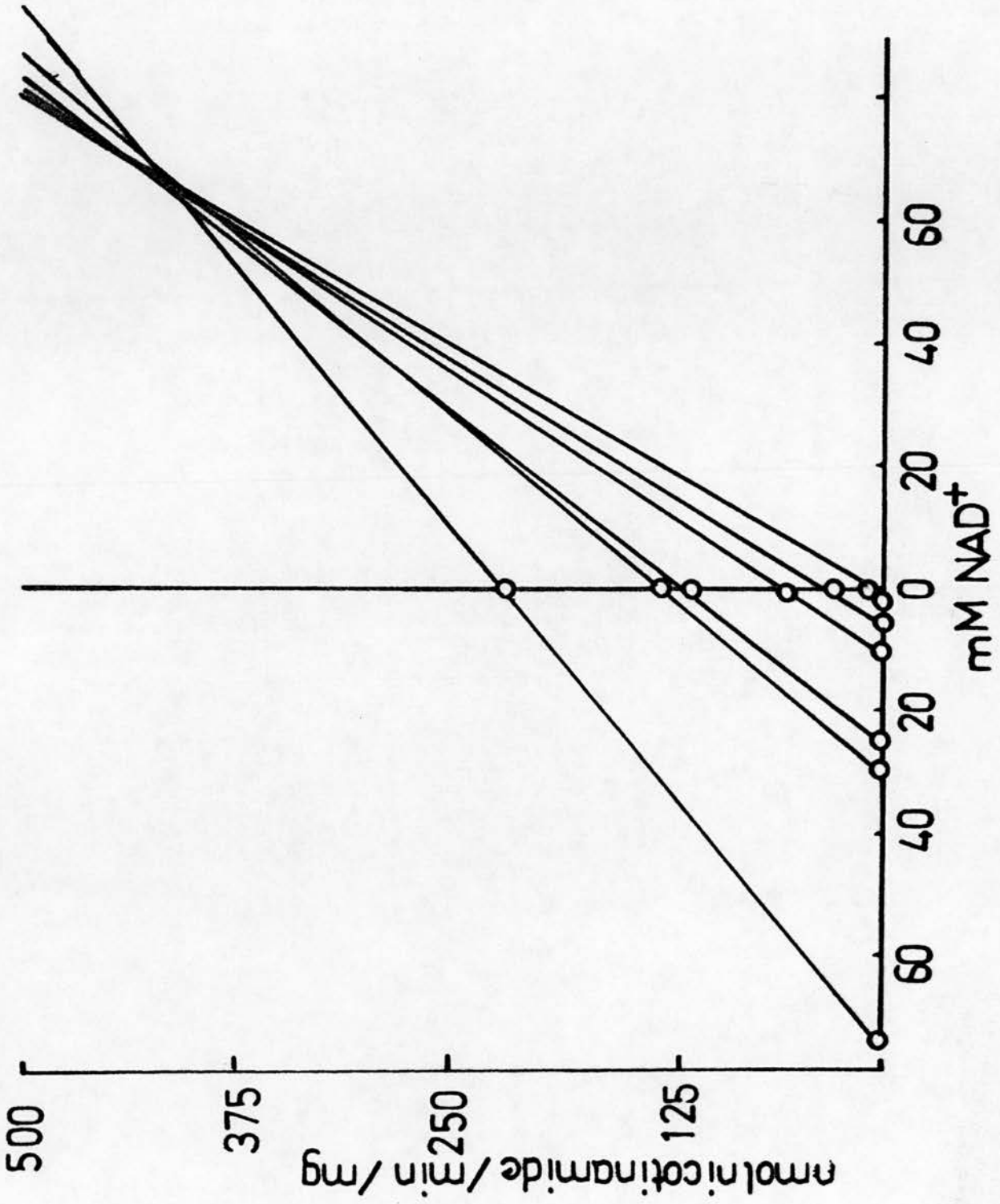

500
375
250
125
nmol nicotinamide /min /mg
60
40
20
0
20
40
60
mM NAD+

fig. 3-8(b)

Numerous attempts were made to obtain sufficient data for a more detailed analysis of the two substrate kinetics of the reaction. However when the concentrations of each substrate were increased, the linearity of the reaction rate was disrupted in a way which suggested product inhibition, substrate inhibition, or both, i.e. the rate fell sharply towards the end of the incubation time. At high concentrations of NAD^+, the NAD'ase activity controls increased substantially. In some cases they were close in value to the sample measurements themselves. At high concentrations of A599 or 4406 the controls for non-enzymic NAD^+ degradation were elevated although not to the same extent as the NAD'ase controls. As well as having adverse effects on enzyme activity and NAD^+ stability, the increased ionic strength caused by the concentrations of each substrate could have been affecting the separation procedure on QA-25 that is the basis of the assay. QA-25 Sephadex is a strongly basic anion exchange resin. At high ionic strength, samples that are normally retained may in some cases pass through the column because of competition from other ions in solution. If NAD^+ were leaking from the columns, this could account for high blank readings (although routine analysis of column effluent on tlc did not reveal large concentrations of NAD^+). In addition to all these problems, another one was presented by the possibility that the arginine derivatives might have been forming micelles. The critical micellar concentration of the derivatives used is not known. In fact nothing is known about their behaviour in solution. The presence or absence of micelles at different concentrations of A599 of 4406 may or may not have affected the assay results.

3.2.7 Competitive inhibition of NAD^+ in the ADP-ribosylation assay

Initial rates of ADP-ribosylation were measured in the presence of fixed levels of a number of partial structural analogues and synthetic derivatives of NAD^+ that contained minor alterations to the adenine or nicotinamide rings. Results obtained using one of these inhibitors, the thionicotinamide derivative of NAD^+ (thio-NAD^+) were analysed using a computer program (Appendix A) and are presented in fig. 3-9 in the form of a double-reciprocal plot. This shows the effect of thio-NAD^+ on the initial rate of ADP-ribosylation at various concentrations of NAD^+. A secondary plot of this data, in fig. 3-10 shows the K_i value for thio-NAD^+ calculated from the results. Similar results were obtained using other NAD^+ derivatives some of which are illustrated in fig. 3-11. Table 3-3 lists the compounds tested and the results obtained. All the compounds tested were competitive with NAD^+. Inhibition was linear which is consistent with the interpretation that competitive binding of these analogues to a single binding site is the cause of the competitive inhibition. The effectiveness of these analogues in inhibiting the reaction should therefore depend primarily on their affinity for cholera toxin, i.e. on the dissociation constant, K_d. This point is explored further in section 5.2.5.3.

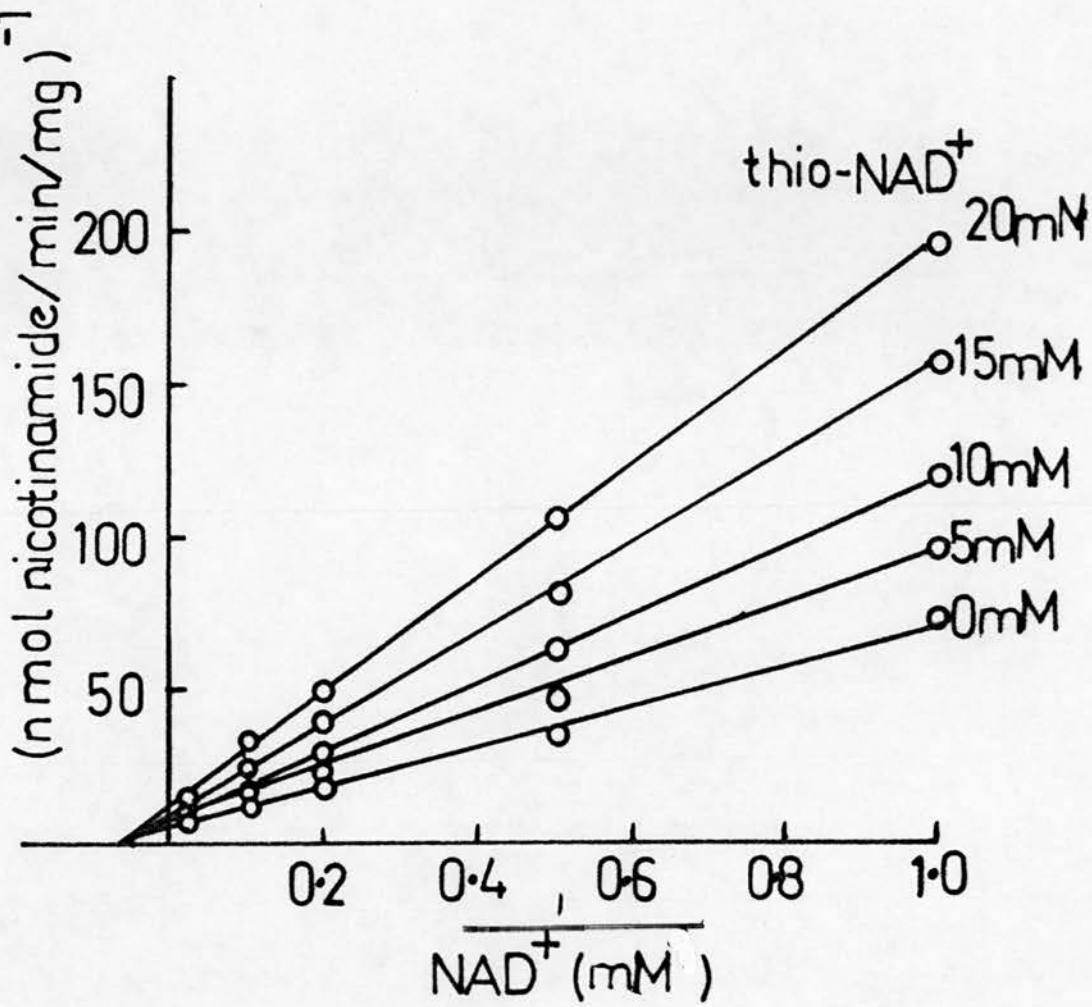


fig. 3-9 Double reciprocal plot of velocity^{-1} versus substrate concentration^{-1} in the presence of the competitive inhibitor thio-NAD$^+$

Assays contained 20mM DTT, 2mM A599, 100µg/ml cholera toxin, 20nCi [4-^{3}H] NAD$^+$ and concentrations of NAD$^+$ and thio-NAD$^+$ as indicated in a total volume of 100µl 200mM KH_2PO_4, pH6.5. Samples were treated as described in Materials and Methods

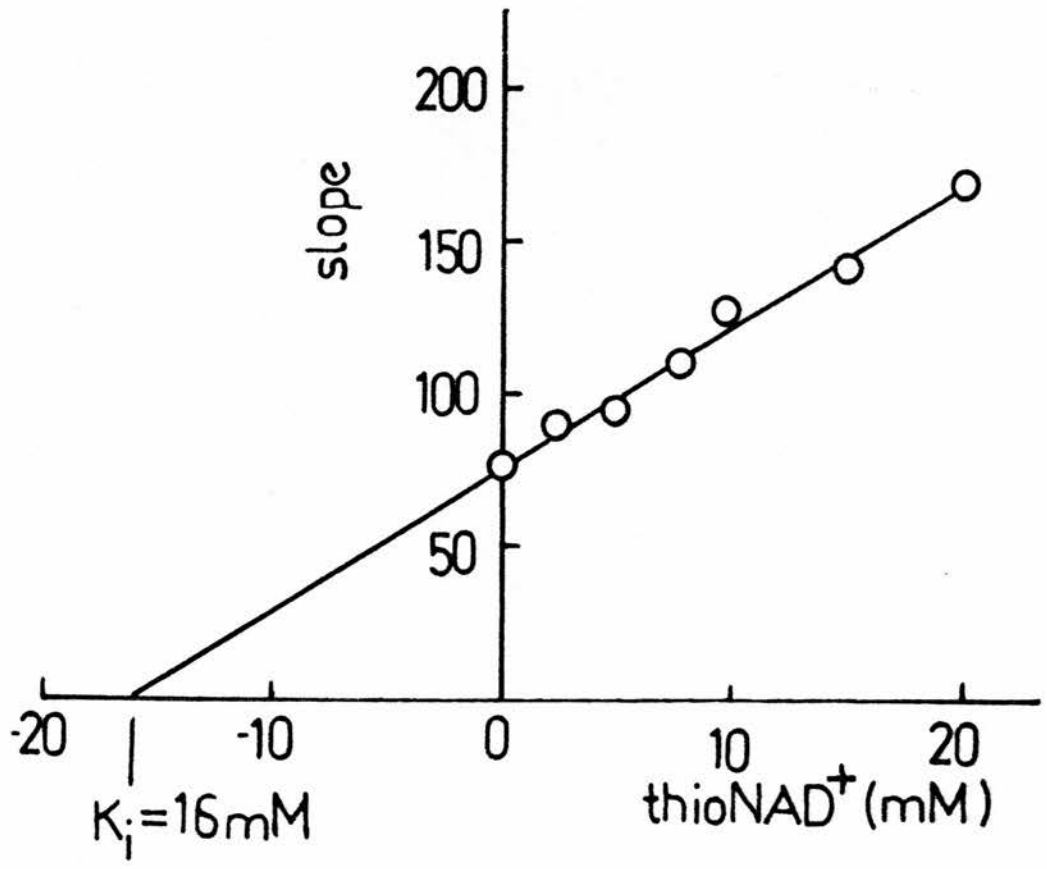
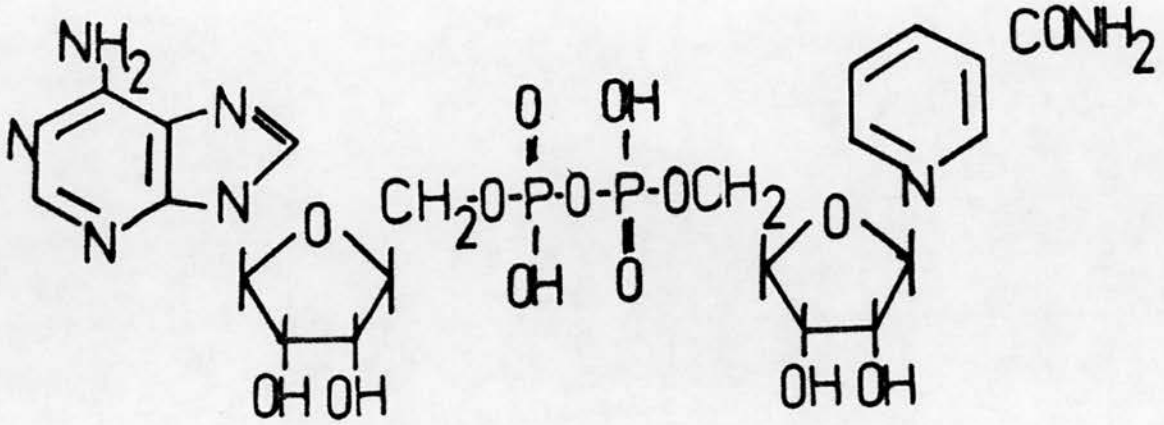
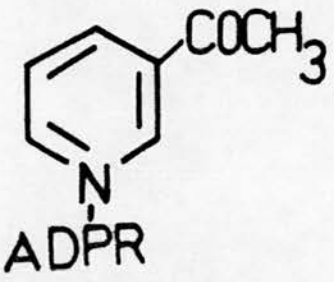


fig. 3-10 Secondary plot of inhibition data

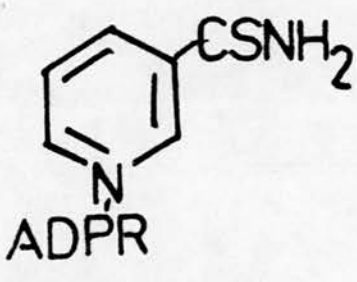
A value of K_i for thio-NAD^+ was calculated using a computer programme described in Appendix A. The results from fig. 3-9 are presented here as a secondary plot of slope (from fig. 3-9) versus inhibitor concentration. The calculated k_i value for thio-NAD^+ was 16mM



NAD+

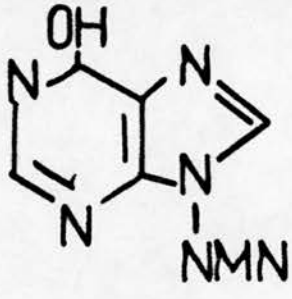


acetyl pyridine adenine dinucleotide (APAD)



thio nicotinamide adenine dinucleotide

3-amino pyridine adenine dinucleotide



nicotinamide hypoxanthine dinucleotide (deamino-NAD+)

fig. 3-11 Structures of NAD+ derivatives

compound	K_i(mM)
thio-NAD^+	16
APAD	17
deamino NAD^+	58
3-amino NAD^+	56
NMN	7100
deamide NAD^+	7100
ADP-ribose	71000
adenosine	71000
adenine	71000
nicotinamide	71000
NADP	N/D
FAD	N/D
benzamide	60
3 aminobenzamide	63

table 3-3 Competitive inhibitors of NAD^+

K_i values were calculated using a computer programme described in Appendix A. Assays were conducted as described for thio-NAD^+ in fig. 3-9.
N/D = not detectable under these conditions

3.2.8 Continuous spectrophotometric assay of ADP-ribosylation

As the indirect assay used had not enabled the two substrate kinetics of cholera toxin's catalytic activity to be studied, a direct assay of ADP-ribosylation activity was sought which might eliminate some of the problems previously encountered. Foremost of these was the fact that in a direct assay of ADP-ribosylation, there is no longer any need to run each and every sample in the presence of a control for NAD'ase activity. One such assay has been recently reported by Soman *et al.*, (1983) who used the guanyl hydrazone p-nitro benzylidine aminoguanidine (pNAG) as a substrate for cholera toxin and other guanidine-specific ADP-ribosyl transferases. This assay has the added advantage of providing a continuous trace of ADP-ribosylation activity. In addition it uses no isotopes. The pNAG is highly coloured with an absorbance maximum at 370nm at pH7.5. After ADP-ribosylation, there is a change in the pK_a value of pNAG which leads to a increase in absorbance at this wavelength. The increase in absorbance may be followed continuously by spectrophotometric means.

Firstly, samples of pNAG were prepared as described in Materials and Methods, which also contains details of the assay procedure. Assay samples contained 40mM DTT, 1 - 10mM NAD^+, 0.4 - 4mM pNAG and 500µg cholera toxin or *E.coli* LT in a total volume of 1ml 20mM tris acetate pH7.5. When LT was used it was incubated with 5µg/ml trypsin for 30 min. at 37°C immediately prior to use and the reaction stopped with 1mM PMSF in propan-1-ol. A time course of the spectral increase at 370nM accompanying the ADP-ribosylation of pNAG is presented in fig. 3-12. This represented a specific activity of

150pmol product/min./mg. The activity seen with LT was too low to be detected. No activity was seen using the isolated A_1 chain. No activity was seen at concentrations of NAD^+ below 10mM. At concentrations of pNAG above 0.4mM, the considerable absorbance of pNAG itself made the assay difficult to follow without the use of a spectrometer sensitive enough to detect accurately a change in absorbance of .05 - 0.1 units per hour against a background of >2.0 units. The pNAG was also increasingly insoluble at concentrations above 0.4mM. It therefore appeared that under the experimental conditions described, there was only a narrow range of NAD^+ and pNAG concentrations which would allow any measurement of ADP-ribosylation activity. No further experiments were performed using this assay system, which even if the problems of solubility were overcome, used huge amounts (500µg) of cholera toxin in each assay. The assay's main advantage, of avoiding expensive radiolabelled NAD^+, was therefore cancelled out by the use of such large amounts of equally expensive toxin.

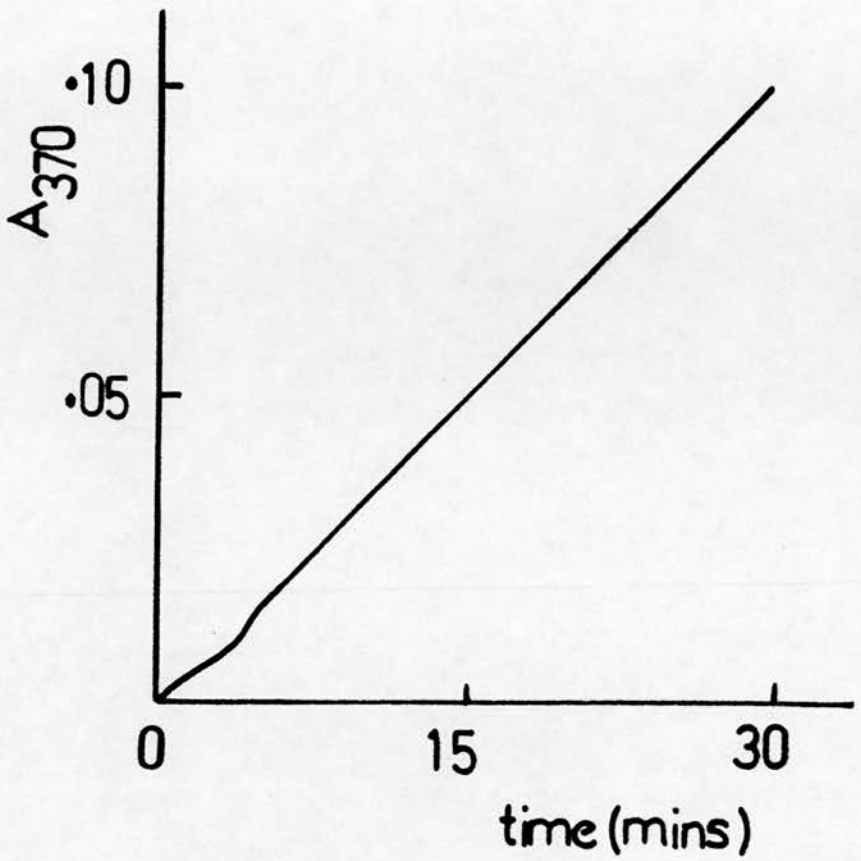


fig. 3-12 Continuous spectrophotometric assay of ADP-ribosylation

Assays were conducted as described in the text. A change in absorbance of 0.1 unit at 370nm was equal to the formation of 5 x 10^{-2}µMol product (Soman *et al.*, 1983)

3.2.9 Retinal transducin as a substrate for cholera toxin

3.2.9.1 Introduction

As mentioned, cholera toxin catalyses the ADP-ribosylation of many proteins in addition to N_s. One of these is transducin, a membrane-bound protein found in the rod outer segments of the vertebrate retina. It couples photolysis of rhodopsin to the activation of a cyclic GMP specific phospho-diesterase (Stryer *et al.*, 1981; Fung *et al.*, 1981) which in turn leads eventually to a nerve impulse in the optic nerve (Miller and Nicol, 1978).

Transducin and N_s and N_i are remarkably similar both in terms of structure and function (Fung, 1983). Transducin is an $\alpha\beta\gamma$ heterotrimer (Manning and Gilman, 1983; Hildebrandt *et al.*, 1984). The subunits of N_s, N_i and transducin cross-react immunologically and the β-subunits at least have largely homologous sequences (Manning and Gilman, 1983). The α subunit of transducin has GTP'ase activity and its ability to activate cyclic GMP phosphodiesterase is controlled by GTP/GDP exchange in a way analogues to the control of N_s and N_i (see Chapter One and fig. 3-13) (Fung and Stryer, 1980, 1980; Fung *et al.*, 1981; Wheeler and Bitensky, 1977). The α subunit can be ADP-ribosylated not just by cholera toxin (Abood *et al.*, 1982) but also by pertussis toxin (Hurley *et al.*, 1984; West *et al.*, 1985). The most striking evidence of the similarity between these G-proteins is the demonstration of functional exchange between components of the two systems such as rhodopsin for hormone-receptor or $N_{i\alpha}$ for transducin-α (Bitensky *et al.*, 1982; Kanaho *et al.*, 1984). Indeed bovine rhodopsin and the mammalian β-adrenergic receptor have themselves been shown to have homologous sequences

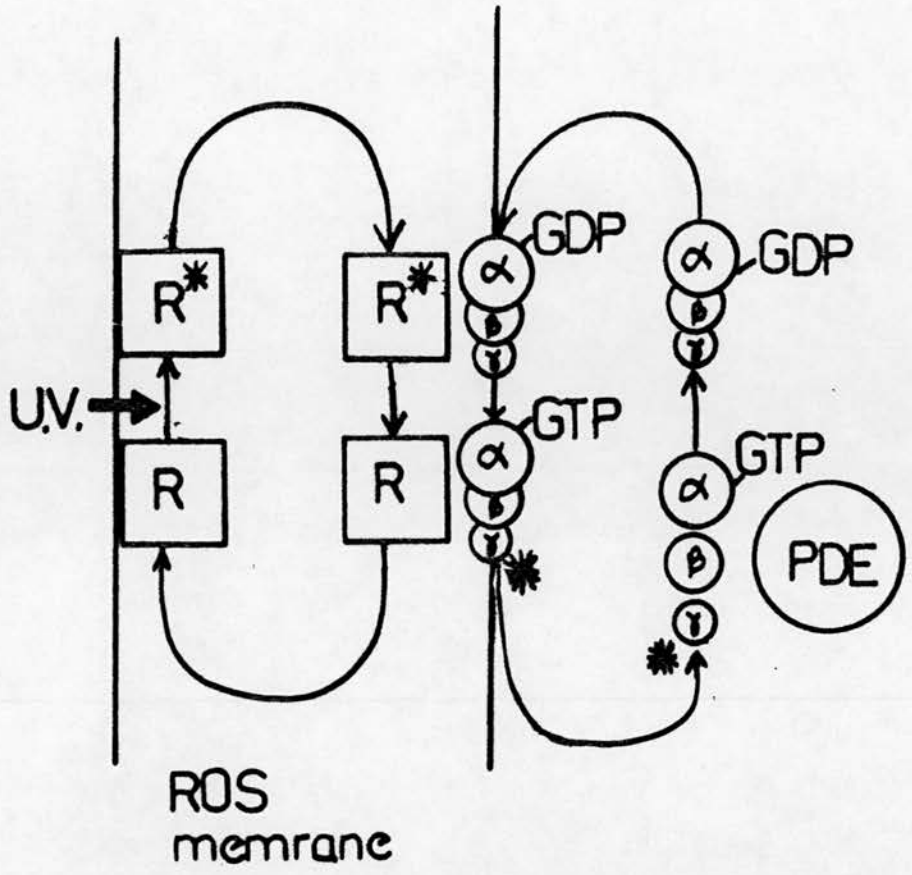


fig. 3-13 A possible mechanism for the interaction of transducin with rhodopsin and the light dependant activation of phosphodiesterase

R represents rhodopsin and R^{*} an active, mobile configuration of rhodopsin formed on exposure to ultraviolet (U.V.) light. Transducin ($\alpha\beta\gamma$, in the presence of photolysed rhodopsin and GTP becomes soluble and may interact with the cyclic GMP phosphodiesterase (PDE) in the cytosol. Hydrolysis of GTP to GDP may return transducin to an inactive conformation.
After Yamazaki *et al.*, (1983)

(Dixon *et al.*, 1986). This demonstrates a remarkable functional compatibility between subunits and implies that specialised peptide domains responsible for protein: protein-interactions have been conserved (Lochrie *et al.*, 1985).

All of this suggests that transducin may be a more readily available alternative to N_s for studying ADP-ribosylation by cholera toxin. Although both proteins are labile and hydrophobic, transducin can be obtained in milligram quantities (Kuhn, 1980). This is because the rod outer segments of the retina are enriched in proteins of the visual transduction pathway and may contain up to 5% transducin compared to the estimate of 0.001% that N_s typically contributes to total cellular protein (Gilman, 1984). Here transducin has been purified and its use as a possible model substrate for N_s investigated.

3.2.9.2 Purification of transducin

The light-induced binding of transducin to rod outer segment (ROS) membranes and its subsequent elution with GTP were used to purify the protein (Kuhn, 1980). Bovine ROS were purified from freshly dissected retinas by flotation and subsequent centrifugation on stepwise sucrose density gradients as described in section 2-6. The ratio $A_{280}:A_{500}$ of the purified ROS was 2.0 - 2.6 indicating good purity with rhodopsin the only protein present (Daeman, 1973). When the purified ROS were analysed on 10% SDS-PAGE, rhodopsin was the major protein present with a band corresponding to molecular weight 34,000 (Godchaux and Zimmerman, 1979). The ROS membranes were exposed to daylight thoroughly and then washed several times to remove soluble proteins. The three polypeptides representing

transducin remained membrane bound and sedimented with the membrane pellet. Transducin was then eluted from the pellet with 40μM GTP in 5mM tris-HCl. The only bands present on 10% SDS-PAGE of this extract corresponded to molecular weights of 39,000 and 36,000 with the 6,000 subunit presumably running with the dye front (fig. 3-14). The yield of purified transducin was approximately 60 - 70μg of each large subunit per mg rhodopsin. Together the two bands made up about 90% of the protein on densitometric analysis of the gel. On average 8mg transducin was purified per 500 bulls eyes.

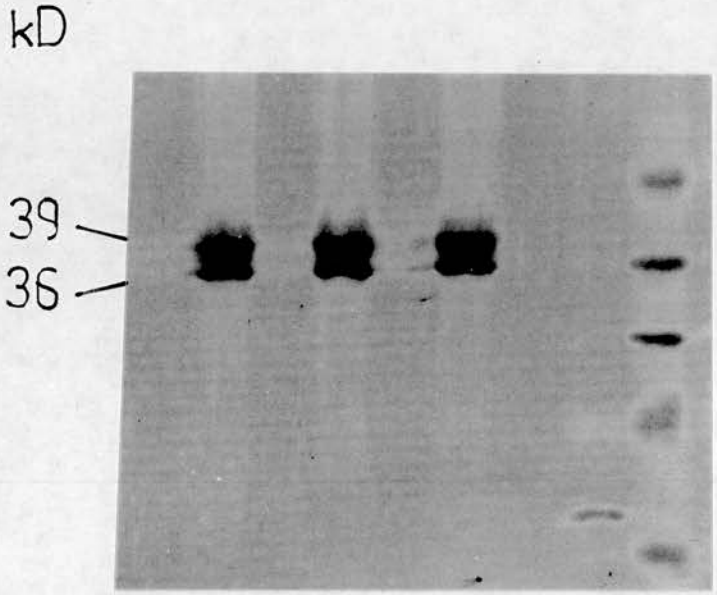


fig. 3-14 Purification of transducin from ROS membranes

Transducin, purified as described in the text, was analysed on 10% SDS-PAGE. The two bands correspond to molecular weights of approximately 39,000 and 36,000

3.2.9.3 Transducin as a substrate for ADP-ribosylation

Van Dop *et al.*, (1984) showed that transducin could be ADP-ribosylated by cholera toxin when still located within ROS membranes. It may only be an efficient substrate however in the presence of photolysed rhodopsin and GTP or Gpp(NH)p (Navon and Fung, 1984). With these points in mind, a portion of the transducin was reconstituted with 'stripped' ROS membranes as described in Materials and Methods.

ROS membranes containing approximately 5mg/ml rhodopsin (100µM) and 0.5mg/ml reconstituted transducin (6µM) were incubated with 50µg/ml cholera toxin, 20mM DTT, 100µM Gpp(NH)p and either [4-^{3}H]NAD^{+} or [U-^{14}C]NAD^{+} (5nCi, or 5 x 10^{5}cpm) in a total volume of 50µl. Some samples contained ROS membranes alone, 2mM A599 or transducin in place of the reconstituted transducin. In some samples cholera toxin was replaced by *E.coli* LT. Samples were incubated for 3 hours at 37°C after which time the samples containing [U-^{14}C]NAD^{+} were subjected to 10% SDS-PAGE. The gel was stained and protein bands cut out and counted. No detectable radioactivity had been incorporated indicating either that no ADP-ribosylation had occurred or that the levels were too low to be detected by this method.

The samples containing [4-^{3}H]-NAD^{+} were treated as follows. An aliquot of each sample (5µl) was analysed using tlc (section 2-10). The remaining 45µl was diluted with 1ml water and separated on QA-25 sephadex as described (section 2.9). Results are presented in table 3-2 as fold stimulation of activity compared to a blank control with no ADP-ribose acceptor. Only the reconstituted

transducin was shown to have stimulated the release of nicotinamide catalysed by cholera toxin, and to a lesser extent by LT. Difficulties arose in quantifying the exact concentration of transducin present in the reconstituted system. The considerable effect of the membranes themselves on the assay in the absence of transducin suggested contamination with unidentified proteins, perhaps NAD'ases. The stability of the transducin preparation was limited possibly due to protease contamination, and the level of activity seen in table 3-2 shows that transducin is only a poor substrate under these conditions.

toxin preparation	A599 acceptor	fold stimulation versus blank
cholera toxin	A599	4.7
	transducin	1.1
	reconstituted transducin	2.4
LT	A599	1.9
	transducin	1.0
	reconstituted transducin	1.4
A_1 chain	A599	1.8
	transducin	1.2
	reconstituted transducin	1.5

table 3-2 ADP-ribosylation activity using transducin as a substrate

Samples were treated as described in the text. When reconstituted transducin was used, the effect of ROS membranes alone was subtracted from the sample value. Results are presented as fold stimulation compared to a blank control identical to the sample but with buffer instead of ADP-ribose acceptor. The transducin/LT experiment is set at 1·0. Each result is the mean of 3, the experiment being repeated twice.

3.3 Discussion

3.3.1 Enzyme activity

These studies provide some additional information about the ADP-ribosylation reaction catalysed by cholera toxin.

The indirect assay using arginine derivatives as ADP-ribose acceptors measured the release of [^{3}H]nicotinamide from [4-^{3}H]-NAD^+. Adequate controls were required to distinguish ADP-ribosylation activity from NAD'ase activity for which water acts as an ADP-ribose acceptor. When the release of [^{3}H] nicotinamide as a function of time was followed under the original conditions described, (2mM A599, 2mM NAD^+), a linear progress curve was obtained. This contrasts with the finding for certain eukaryotic ADP-ribosyl transferases of biphasic kinetics for NAD^+ (Furneaux and Pearson, 1980). For the eukaryotic enzymes, this was taken as an indication either of substrate activation by NAD^+ or of multiple binding sites for NAD^+ on the toxin.

A calculated value of 3mM for NAD^+ was observed for cholera toxin's NAD'ase activity (i.e. with no A599 or 4406 present). This value agrees well with published results (Mekalonos *et al.*, 1979a; Moss *et al.*, 1979b). When the arginine derivatives A599 or 4406 were included in the assay, the K_m value for NAD^+ rose almost tenfold to 45 - 51mM. A similar result was reported by Osborne *et al.*, (1985) when they studied the toxin using agmatine as an acceptor for ADP-ribose. They showed that binding of either NAD^+ or the arginine substrate had a negative effect on the subsequent binding of the other substrate. They proposed a random order rapid equilibrium model for cholera toxin and accounted for the reduced

affinities as a possible control mechanism to allow certain arginine residues to be more readily ADP-ribosylated than others.

These high K_m values for NAD^+ contrast markedly with the micromolar K_m values for NAD^+ reported for most other ADP-ribosyl transferases such as diphtheria toxin (Kandel *et al.*, 1974) pertussis toxin, (Moss *et al.*, 1983). *P.aeruginosa* exotoxin A (Chung and Collier, 1977b) and at least one mammalian ADP-ribosyl transferase (Burtscher *et al.*, 1986). The cellular concentration of NAD^+ is about 70μM in pigeon erythrocytes (Gill, 1975) and is unlikely to exceed 100μM in any cell type. This makes the millimolar K_m of cholera toxin seem inexplicably high. However, Tait (1981) showed that for optimal activation of adenylate cyclase in rat liver membranes, cholera toxin required the addition of 1mM exogenous NAD^+. In this case high NAD^+ concentrations were required in an assay system which was more closely analogous to the *in vivo* situation than the one used here. Membrane-bound N_s was the substrate for ADP-ribosylation and the various factors reported to be necessary for cholera toxin's activity *in vivo* would have been present. These include an unidentified cytosolic factor of molecular weight 16000 (Enomoto and Gill, 1979) and an intrinsic membrane protein of molecular weight 21000 called ARF, which has been purified by Kahn and Gilman (1984). Their results imply that the substrates for cholera toxin in rat liver membranes are NAD^+ and a GTP-N_s-ARF complex and the reaction proceeds in a lipid environment. Their assay system required the addition of only 100μM exogenous NAD^+ in order to demonstrate a significant level of ADP-ribosylation. Gill and Woolkalis (1985), compared various

substrates for ADP-ribosylation by cholera toxin and showed that N_s was by far the preferred substrate when compared to a number of proteins and arginine derivatives. There must be a rather special environment of the relevant arginine in N_s that makes it so susceptible to ADP-ribosylation, an environment which none of the alternative substrates considered in this Chapter; A599, 4406, pNAG or transducin is providing. In conclusion then, cholera toxin has an extremely high K_m for NAD^+. This is reflected in the K_d value calculated in Chapter 5, indicating a low affinity binding of NAD^+ to the toxin. Explanations for this high K_m include the possibility that NAD^+ is not the true substrate *in vivo*, but as most derivatives and breakdown products of NAD^+ have been examined, an alternative to NAD^+ would have to be an as yet unidentified derivative.

Alternatively, there could be an intracellular pool of NAD^+ providing high localised concentrations although there are no results to suggest that this is the case.

It could be that cholera toxin's possible membrane location when it catalyses its ADP-ribosylation reaction has some bearing on the availability of its substrates *in vivo*.

3.3.2 A599, 4406 and the indirect assay

The presence of A599 or 4406 seemed to have a negative effect on the binding of NAD^+ to the toxin. The *in vivo* substrate N_s is a large intrinsic membrane protein (Kaslow *et al.*, 1980). Its high frictional ratio and the small amount of detergent it binds after solubilisation suggest it to be elongated and anchored into the membrane by a hydrophobic stalk (Brunner *et al.*, 1979). As mentioned, the actual substrate for ADP-ribosylation by cholera

toxin *in vivo* may consist of N_s with a number of interacting cofactors, with ADP-ribosylation sites within the membrane environment (Johnson, 1981). In contrast to this complex situation, A599 is a small guanidine derivative with a polar head group separated from a charged end group by an unbroken length of hydocarbon chain. This hydrophobic chain seems to be an important structural prerequisite in making A599 a better substrate for cholera toxin than arginine alone (Tait and Nassau, 1984) and may mimic the susceptible arginine(s) on N_s. Assuming that either A599 or NAD^+ can bind to toxin first, it could be that A599 can bind and can act as a substrate, but cannot induce a conformational change (whch N_s presumably can). This change in conformation may be needed to form a high affinity binding site for NAD^+. A599 would then be an unproductive substitute for N_s. Alternatively, a small molecule like A599 could have access to inappropriate sites on cholera toxin which are blocked to a large protein like N_s. These sites may then interfere with the subsequent binding of NAD^+.

Whatever the reasons, the indirect measurement of nicotinamide release did not seem to be an adequate method for studying the kinetic mechanism. When concentrations of NAD^+ and more especially of A599 were varied, the linearity of the reaction was disrupted in a way that suggested substrate inhibition. The high levels of apparent NAD^+ hydrolysis seen in the blank controls were in some cases similar to the actual samples. The NAD'ase activity of cholera toxin has been reported to be greater relative to ADP-ribosylation activity at high NAD^+ concentrations and could be due in some cases to contamination (van Heyningen, 1977a; Tait and

van Heyningen, 1980). A direct assay such as the one described by Mekalonos *et al.*, (1979a) using ^{125}I-guanyl tyramine seems more appropriate for these kinds of measurements.

3.3.3 Transducin as a substrate

In neither of the forms studied; pure or reconstituted, was transducin an efficient substrate for cholera toxin. The activity of the reconstituted transducin in a GTP'ase assay was not checked, and its correct orientation within the membrane was therefore not established. Even so, it seems that transducin, despite its striking similarities to N_s, is not as good a substrate for cholera toxin (see table 3-2). In fact transducin appears to be a better model for N_i. Peptide mapping has shown that transducin's α subunit is more closely homologous to $N_{i\alpha}$ than to $N_{s\alpha}$ (Manning and Gilman, 1983). The homology between $N_{i\alpha}$ and transducin-α is accentuated by the fact that both are substrates for ADP-ribosylation by both pertussis toxin and cholera toxin whereas $N_{s\alpha}$ is a substrate only of cholera toxin (Owens *et al.*, 1985). If transducin were to be used as a model for N_s, a direct assay, perhaps using [^{32}-P]-NAD$^+$ would be more suitable than the indirect assay here. Again, problems with high control values designed to detect NAD'ase activity were found. The membrane preparations could very well have had intrinsic NAD'ase activity due to contaminating proteins.

3.3.4 Cholera toxin's kinetic mechanism

The results from the indirect assay did not provide sufficient information to say what the kinetic mechanism of cholera toxin is likely to be. However, the results obtained, together with binding results from Chapter 5 can be compared to previously published

results. There is evidence from both Osborne *et al.*, (1985) and Mekalonos *et al.*, (1979a) that the reaction proceeds through a ternary complex. Assuming that this is so, there are then two possible mechanisms, either ordered or random sequential binding of substrates. Both have been proposed. As outlined previously, Osborne's model was of a random order rapid equilibrium mechanism, with binding of the first substrate having an effect on the subsequent binding of the next substrate which for agmatine was negative but which for the *in vivo* situation may even be positive. Alternatively, Mekalanos proposed an ordered sequential model, with NAD^+ binding first.

A key point in comparing these models is whether or not the ADP-ribose acceptor, be it ^{125}I-guanyl tyramine, A599 or N_∞, can bind to cholera toxin in the absence of NAD^+. Results from binding studies in Chapter 5 show that although no binding of A599 to cholera toxin was detected using a number of methods (including changes in absorbance or fluorescence) an effect on the toxin's conformation caused by A603 was seen in section 5.2.5. It was shown that saturating concentrations of A603 (another arginine derivative, illustrated in fig. 3-1) could protect the A_1 chain of the toxin from digestion with trypsin. This was interpreted as evidence that A603 was binding to the A_1 chain and protecting it from digestion through a change in conformation. Taken together with the results from this Chapter which suggest that the affinity of the toxin for NAD^+ is reduced in the presence of A599, Osborne's proposed random order rapid equilibrium model seems the most likely mechanism for cholera toxin. This model is illustrated in fig. 3-15.

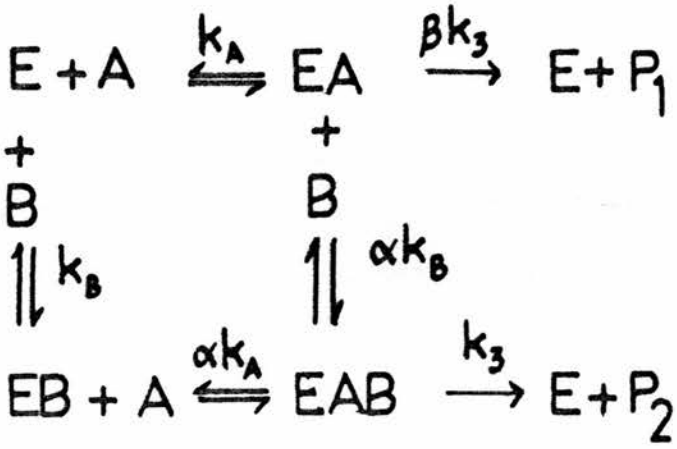


fig. 3-15 A possible kinetic mechanism for cholera toxin

In this mechanism, the binding of one substrate alters the binding of the other substrate by a factor α. If $\alpha<1$, the affinity for the second substrate is increased, whereas if $\alpha>1$ it is decreased. The apparent maximal velocity changes when either A or B serves as the variable substrate. A and B correspond to NAD^+ and A599 respectively and the equilibrium dissociation constants are given by k_A and K_B. The factor β is equal to the ratio of maximal velocities of NAD^+ Glycohydrolase and transferase reactions.
From Osborne *et al.*, (1985)

It would be interesting to see whether this mechanism is also seen for *E.coli* LT, which seems to be similar to cholera toxin in so many ways (Chapter 4). Osborne's work shows that a turkey erythrocyte ADP-ribosyl transferase studied at the same time as cholera toxin had an identical mechanism, although its K_m for NAD^+ was only 7μM. It appears that cholera toxin differs from other toxins such as diphtheria toxin and exotoxin A, not only in its high K_m of approximately 4mM (elevated to around 50mM under certain assay conditions), and the non-specific nature of its ADP-ribosylation, but also in having a random order mechanism. Diphtheria toxin and exotoxin A have compulsory order mechanisms (Chung and Collier, 1977b). They are both entirely specific for EF2, possibly because the site of modification, a 'diphthamide' residue, has not been found elsewhere. Cholera toxin could turn out to be more closely related to other arginine-specific ADP-ribosyl transferases, both bacterial and eukaryotic (see fig. 1-6) than to the diphthamide-specific bacterial toxins.

3.3.5 Inhibitor Studies

The only competitive inhibitors of NAD^+ of any significance were those that contained all of the NAD^+ molecule with minor alterations to the nicotinamide ring. Some, such as thio-NAD^+ can substitute for NAD^+ in most NAD^+-requiring reactions, while others such as acetyl pyridine adenine dinucleotide (APAD) are replacements for NAD^+ for many dehydrogenases. The other molecules tested, which included NADP, FAD, NADH, purine nucloeotides, nucleosides or bases were not significant inhibitors. Gill (1976a) reported that only thio-NAD^+ could replace NAD^+ for the activation of erythrocyte

adenylate cyclase by cholera toxin.

As shown in table 3-3, the most efficient inhibitors were thio-NAD^+ with a sulphur group at carbon 3 of the nicotinamide ring and APAD with a C3 acetyl. Both deamido-NAD^+ with a C3 carboxylic acid and 3-amino NAD^+ with a C3 amino group were poor inhibitors. There appears to be some correlation between the C3 substituent of the nicotinamide and that molecule's efficiency as an inhibitor. Presumably the C3 substituent is involved in some specific interaction at the binding site, perhaps a hydrogen bond, which cannot be achieved, e.g. by the carboxylic acid derivative, deamido-NAD^+. The nicotinamide ring as a whole plays an important role in binding as shown by the inhibitory effects of nicotinamide itself and of analogues such as benzamide. Although compounds lacking the nicotinamide ring were in general poor inhibitors, e.g. AMP, adenine, the adenine moiety does have some importance in binding. Deamino-NAD^+, in which the amino group of adenine is replaced by a hydroxyl group was a poor inhibitor. Presumably the hydroxyl group caused electrostatic repulsion.

Benzamide and 3-amino benzamide had some inhibitory activity. These compounds are potent inhibitors of eukaryotic ADP-ribosyl transferases (Purnell *et al.*, 1980) and have even been used in the preparation of affinity chromatography media for their purification (Burtscher *et al.*, 1986). Although this might point to homologies between eukaryotic enzymes and cholera toxin, benzamide is most probably recognising a general NAD^+-binding region, as other enzymes involved in NAD^+-metabolism are also inhibited by benzamide (Johnson, 1981). Diphtheria toxin and exotoxin A have similar

sensitivities to inhibition (Chung and Collier, 1977) and indeed have almost identical enzyme activities but are not particularly homologous in their amino acid sequences. Although cholera toxin's active site may share some features with these two enzymes, any similarities are likely to be confined to the NAD^+-binding portion of the active site. The other part is more likely to resemble arginine-specific enzymes. Certainly the phenomenon reported for both diphtheria toxin and exotoxin A in which adenine was a more potent inhibitor than adenosine which in turn was better than ADP-ribose (Chung and Collier, 1977) was not apparent for cholera toxin (although the inhibition was so slight as to be difficult to quantify at all). Presumably, entropy factors were responsible for this decrease in affinity.

Chapter 4 Purification of E.coli heat labile toxin (LT)

4.1 Introduction

Certain strains of *E.coli* associated with enteropathogenic disease in man and other animals are known to produce a diarrhoea-inducing exo enterotoxin similar in many respects to cholera toxin (Finkelstein, 1973). *E.coli* heat labile toxin (or LT as it is commonly called) has the same subunit structure as cholera toxin with one toxin-active A subunit and five receptor binding B subunits. The B subunits are arranged in a ring and held together by tight non-covalent bonds. The A subunit is linked to and partially inserted in the B ring through weaker non-covalent interactions (Clements and Finkelstein, 1979; Gill *et al.*, 1981). Unlike cholera toxin, the A subunit of LT is normally purified un-nicked. Subsequent treatment with trypsin nicks the LT A subunit and increases the biological activity of the LT molecule (Rappaport *et al.*, 1976) probably by cleavage and removal of two amino acid residues after arginine 188 (Spicer and Noble, 1982).

The two toxins are functionally almost identical. The receptors for both cholera toxin and LT in the gut mucosa are G_{M1} gangliosides although the receptor for LT may also contain a structurally related glyco-protein (Holmgren *et al.*, 1982). The A_1 chain of LT can catalyse the same enzyme reactions (Moss *et al.*, 1979a) and ADP-ribosylations (Gill and Richardson, 1980) as those of cholera toxin, resulting *in vivo* in a similar activation of adenylate cyclase. Both subunits cross-react immunologically (Moseley and Falkow, 1980) although each has unique determinants. Both cholera toxin and LT are encoded by polycistronic operons but whereas cholera toxin's ctx

operon is chromosomally encoded, the *elt* operon for LT is located on a variety of plasmids (Gyles *et al.*, 1974; So *et al.*, 1978) and even bacteriophages (Takeda and Murphy, 1978) harboured by entero pathogenic strains of *E.coli*. Analysis of the nucleotide sequence of the *elt* genes suggests that they are cotranscribed from a typical *E.coli* promoter preceding the *elt* A gene (Rosenberg and Court, 1979) and that *elt* mRNA contains two Shine-Dalgarno sequences (Steitz, 1979) preceding the AUG translation starts for LT A and LT B. The Shine-Dalgarno sequence of LT B resembles that of cholera toxin B in that it is more closely homologous to 16SrRNA than that of the A subunit. Greater translational efficiency would then explain the production of five B subunits for every A subunit.

Comparison of the amino acid sequences of the two toxins shows that they are homologous to varying degrees throughout their primary structures. Of the 103 residues of the LT B subunit only 22 differ from the corresponding residues in cholera toxin and in 20 of these a single base-pair change in the *elt* B codon could account for the differences (Dallas and Falkow, 1980). The LT A subunit contains 236 amino acids and shows a high degree of homology to cholera toxin A, particularly the A_1 chain (Spicer and Noble, 1982). A more recent sequence analysis (Dykes *et al.*, 1985) reports even greater homology between LT and cholera toxin of 80% overall. Yamamoto *et al.*, (1984) have compared the available nucleotide sequences of cholera toxin and of porcine and human LT strains. They showed that in addition to their homologous sequences, both codon usage and G+C content of the nucleotide sequences were the same. Amino acid homology was most apparent in the A_1 chains which contained four

stretches of identical sequence. A short stretch of high divergence near the end of the LT A_1 chain had a unique base change pattern. Divergence in this region may be the result of adaptation to the different proteases of *V.Cholerae* and *E.coli*. Secondary structure predictions place this sequence on a hydrophilic region of random coil, which may be looped out on the surface of the toxin (Yamamoto *et al.*, 1984). The porcine LT showed greater divergence from cholera toxin than did human LT. Much of this divergence was compressed within a short stretch of the A_1 chain. The reason for this is unclear but could represent a species-specific alteration.

These observations raise interesting questions regarding the similarity of the two enterotoxins. For instance, if specific regions are strongly conserved, are they associated with special functions such as NAD^+ binding, ADP-ribosylation of adenylate cyclase, the formation of bonds between A and B subunits and the movement of the A subunit across the cell membrane into the target cell?

In this Chapter, the purification and characterisation of porcine LT is described. The purification relies on the ability of LT to adhere to columns of agarose from which it can be eluted by galactose (a monomer component of agarose), in practically homogenous form. The specific binding affinity of LT B for plastic adsorbed G_{M1} ganglioside is used in an ELISA procedure to detect LT during the purification. This work was done in the Department of Microbial Biochemistry, Glaxo Group Research Ltd.

4.2 Results

4.2.1 Purification and characterisation of LT

In this study fractions were screened not for biological activity but for antigenic activity. The strain of *E.coli* was *E.coli* 2107E (containing plasmid EDW299 (porcine LT A^+ B^+ ap^r)) and was provided by Glaxo Group Research Ltd. It is a genetically engineered strain which produces 50 - 100 fold more toxin than do normal strains (Evans *et al.*, 1974). About 1% of its total cellular protein or up to 20% of its periplasmic protein is enterotoxin. Cells were harvested and lysed by sonication. After precipitation with 60% $(NH_4)_2SO_4$ preparations were applied to a column of Bio-Gel agarose A5 m. A typical elution profile from the agarose column is shown in fig. 4-1. The G_{M1} ELISA revealed that little or no LT eluted from the column with TEAN buffer (50mM tris-HCl, 200mM NaCl, 3mM NaN_3, 1mM EDTA, pH7.5. After 0.2M galactose was applied, a single ELISA positive peak eluted with the leading edge of the applied galactose. This peak, when pooled, concentrated by ultrafiltration on Amicon P10 filters and analysed by 15% SDS-PAGE, consisted of two non-covalently associated subunits (fig. 4-2). Of these, the slower migrating component corresponded to a protein of molecular weight 28,000, the same as cholera toxin A subunit.

Treatment with thiol (which separates cholera toxin A subunit into its A_1 and A_2 peptides) had no effect upon the apparent molecular weight of this band, indicating that the LT A is in a completely un-nicked form. Treatment of the purified LT with trypsin before thiol reduction and electrophoresis resulted in complete dissociation of LT A into its A_1 peptide of molecular weight 21,000 and its A_2

peptide of molecular weight 6,000 (and not visible on the gel in fig. 4-3) The biological activity of LT samples treated with concentrations of trypsin from 1 - 20 µg/ml using the ADP-ribosylation assay described in section 2.9 reveals that there is an optimum concentration of trypsin for enhancing the ADP-ribosylation activity of the toxin (fig. 4-3).

4.2.2 ADP-ribosylation activity of LT and of cholera toxin

The activities of cholera toxin and of the purified LT were compared using the ADP-ribosylation assay (section 2.9). The LT was treated with 5µg/ml trypsin for 30 min. at 37°C immediately prior to use. The digestion was stopped with 1mM PMSF in propan-1-ol. Samples of cholera toxin and nicked or unnicked LT at concentrations in the range 50-500µg/ml (6-60µM) were compared. Controls were included to check that PMSF, propan-1-ol and trypsin did not affect the assay system. The nicked LT consistently showed less than half the activity of cholera toxin in this concentration range; the un-nicked LT showing no activity at all (fig. 4-4). Both toxins are able to use A599 as a substrate as reported previously (Tait, personal communication).

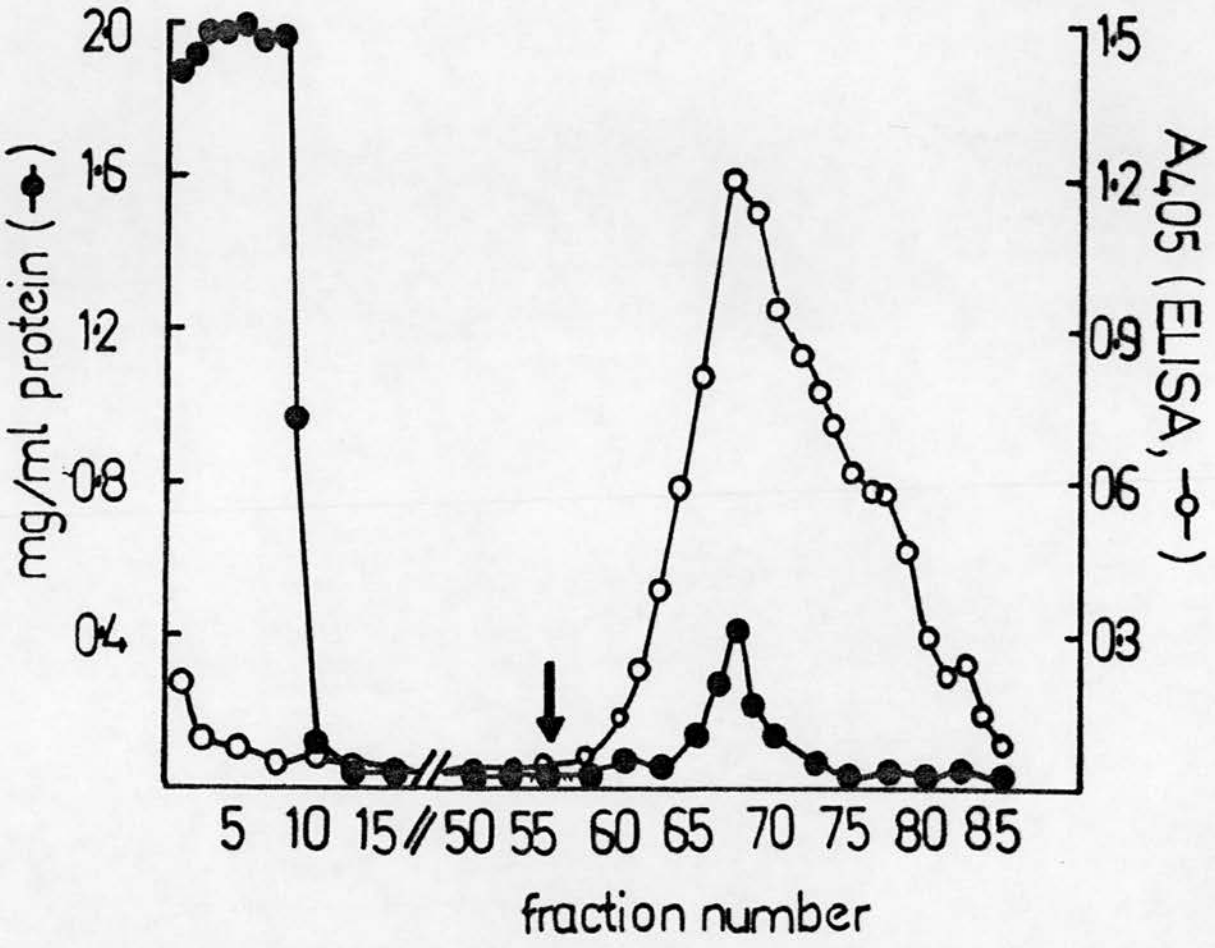


fig. 4-1 Gel filtration chromatography of crude LT on Bio-Gel A5m (2.5 by 80 cm)

The flow rate was 20ml/h and fractions of 10ml (up to fraction 15) then 5ml were collected. The arrow marks the start of the galactose (0.2M) wash

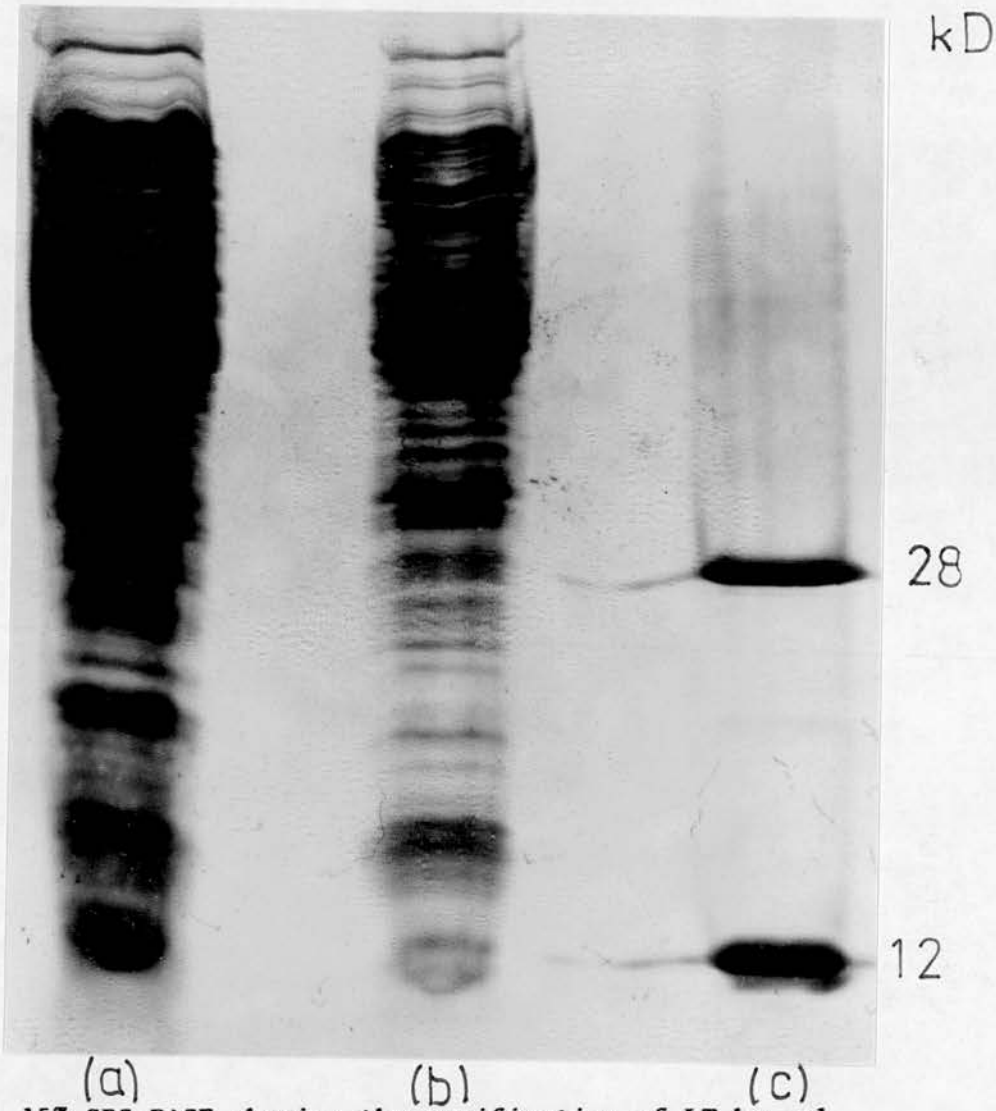


fig. 4-2 15% SDS-PAGE showing the purification of LT by gel filtration on Bio Gel A5m agarose

Lane (a) shows the crude $(NH_4)_2SO_4$ precipitate which was loaded onto the Bio Gel A5m column. Lane (b) shows the protein eluted with TEAN buffer wash. Lane (c) shows LT eluted with galactose

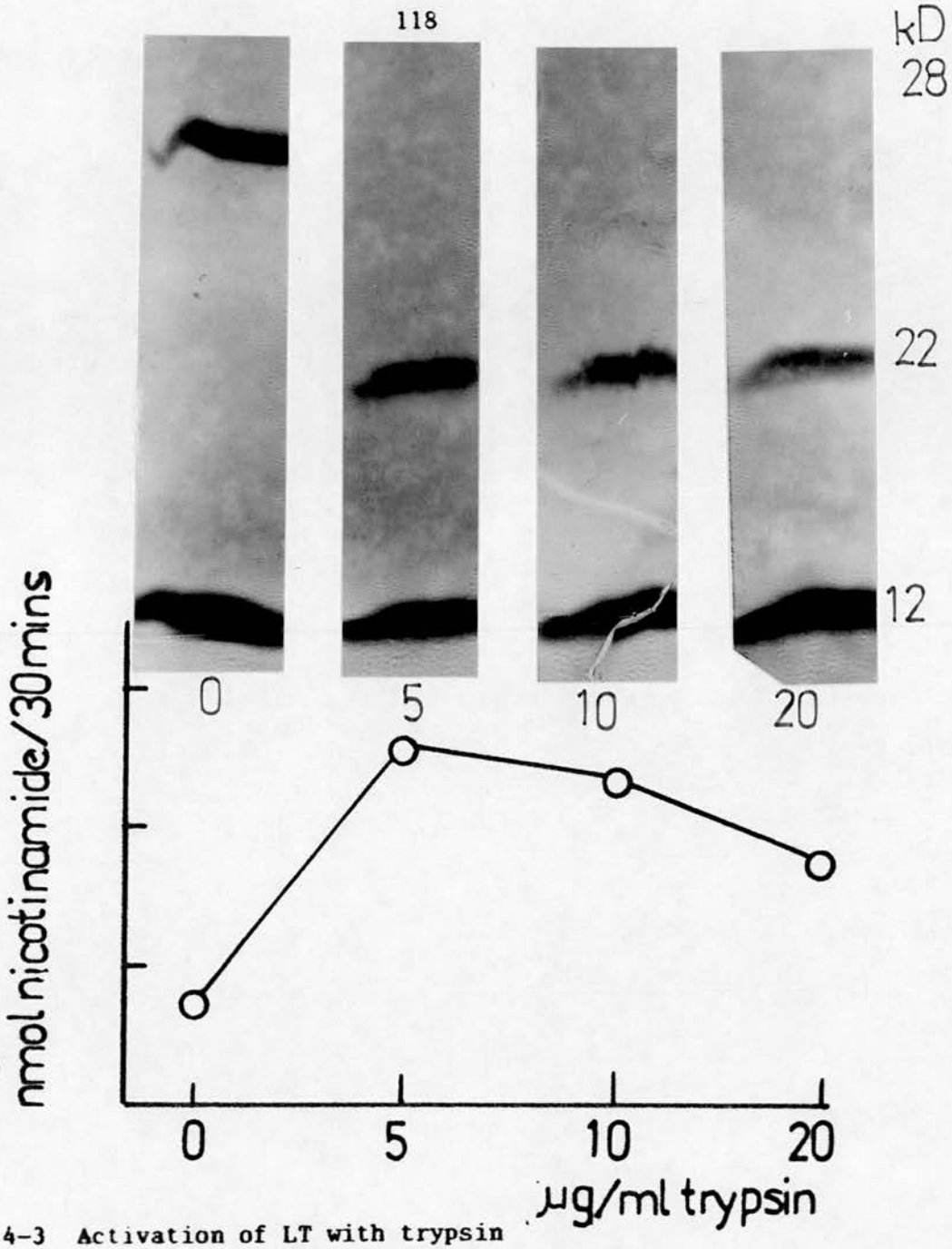


fig. 4-3 Activation of LT with trypsin

Samples of LT (400µg) were incubated with 0 - 20 µg/ml trypsin. 20mM DTT in a total volume of 150µl KH_2PO_4, pH6.5. After 30 min. at 37°C, reactions were stopped with PMSF. 100µl of each sample was analysed by 15% SDS-PAGE. The remaining 50µl was assayed using the ADP-ribosylation assay described. Photos represent the protein sample obtained at each trypsin concentration.

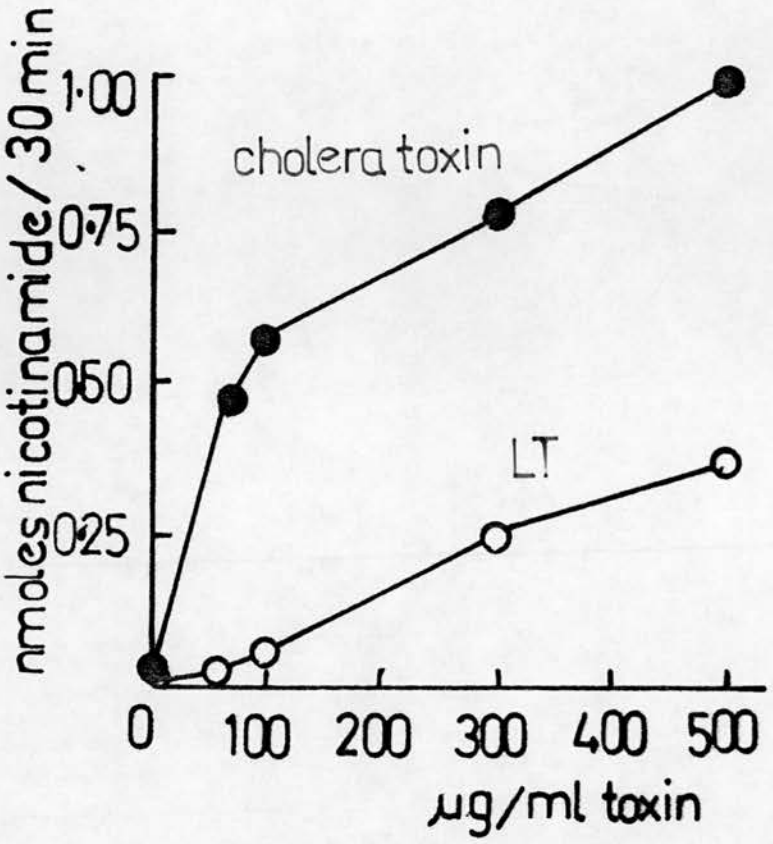


fig. 4-4 ADP-ribosylation activity of cholera toxin and of LT

Purified LT (4mg/ml) was treated with 5µg/ml trypsin as described. A similar sample of cholera toxin was treated in an identical way but without adding trypsin. Both toxins were diluted to give a range of 5 - 500µg/ml by the addition of a cocktail of 2mM NAD^+, 20nCi [$4^{-3}H$]NAD^+ and 2mM A603 in a total volume of 100µl KH_2PO_4, pH6.5. Samples were treated as described in Materials and Methods

4.2.3 Fluorescent labelling of LT with Nbf-Cl

The interaction of LT with NAD^+ was studied using LT which had been fluorescently labelled with Nbf-Cl (4-chloro-7 nitrobenzofuran). A solution of LT was pretreated with 5µg/ml trypsin for 30 min. at 37°C in the presence of 5mM DTT, the digestion being stopped by the addition of 1mM PMSF in propan-1-ol. Solutions of either this nicked LT or of untreated LT were then labelled with Nbf by incubating with a near saturating solution of Nbf Cl at pH8.5 a described in section 2.13 (see also section 5.2.4.1). After this treatment, a portion of the un-nicked, labelled toxin was then treated with trypsin as described above. This gave three different preparations: un-nicked, Nbf- labelled LT; LT nicked before labelling and LT nicked after labelling. When these samples were analysed by 12% SDS-PAGE they showed three bands (two for the un-nicked LT) visible under ultraviolet light and staining with coomassie blue with the expected mobilities of subunits A, B, A_1 or A_2. Measurement of the absorbance for protein at 280nm and for the amino derivative of Nbf at 475nm suggested that about two moles of Nbf had bound per mole of toxin in each case.

When solutions of the three Nbf-labelled toxins were treated with NAD^+ (section 2.13) there was no alteration in either the fluorescence intensity or the wavelength of maximum intensity which stayed at 533nm. This was in contrast to the results obtained using Nbf-labelled cholera toxin A_1 chain for which a marked enhancement of fluorescence was seen on addition of NAD^+ as outlined in section 5.2.4.2.

4.3 Discussion

These studies verify previous reports which suggest that although LT and cholera toxin have remarkable physiochemical similarities, each possesses unique features. The purification procedure used highlights some of these differences. Bio Gel agarose A5m is acting here as a kind of affinity matrix for LT. This is not altogether surprising since LT, like cholera toxin, interacts with G_{M1} ganglioside of which galactose is a component. However LT differs from cholera toxin which although retarded is not prevented from elution on agarose (Finkelstein and Lospalluto, 1969). This difference may reflect differences in the nature of the interaction of the two toxins with host cell membrane receptors. The LT A subunit has an unusually high content of charged residues: 24 arginine, 17 aspartate, 15 asparagine and 14 glutamate. It also has remarkably high concentrations of tyrosine (21) and phenylalanine (11) (Spicer and Noble, 1982) which may account for the strong affinity for agarose (Clements and Finkelstein, 1979). Cholera toxin is similar in having a high content of charged residues but is richer in glutamate, glutamine, aspartate and glycine residues than is LT.

The purification procedure uses a whole-cell lysate of LT as, unlike cholera toxin which is mostly secreted by *V.Cholerae*, LT is predominantly intracellular in *E.coli* and little is found extracellularly. A variety of Gram negative bacteria including *Vibrios* and *Pseudomonas* species secrete proteins through their cell envelopes into the extracellular environment. Other Gram negative bacteria including *E.coli* rarely exhibited this phenomonen and may

lack the secretory apparatus to do so. Hirst *et al.*, (1984) described the synthesis of LT by both *E.coli* and *V.Cholerae* strains engineered to express the plasmid encoded *elt* operon but not the *ctx* operon. They demonstrated that while *E.coli* retained LT in a cell-associated compartment, *V.Cholerae* secreted it into the extracellular milieu. Thus LT is exported to two different locations depending on the organism in which it is synthesised, suggesting that bacterial-physiologial factors as well as the structural properties of LT are important in determining its location.

The LT purified from *E.coli* 2107E was in a completely un-nicked form. The nicking of the A subunit may be relevant to toxin secretion because cholera toxin has previously been shown to have nicked A subunits when secreted from *V.Cholerae* (Lönnroth and Holmgren, 1973) but un-nicked A subunits when cell-associated in *E.coli* (Pearson and Mekalonos, 1982). However, Hirst *et al.*, (1984) show that nicking must be important in the secretory process because both porcine and human LT were completely secreted from Vibrio Cholerae without their A subunits being nicked. The *V.Cholerae* protease that nicks cholera toxin has been proposed to be soluble hemagglutinin/protease which can also nick LT *in vitro* (Finkelstein *et al.*, 1983). The *V.Cholerae* strain used by Hirst *et al.*, TRH 7000, presumably produced insufficient hemagglutinin to nick the LT. The relevance of LT's intracellular location and un-nicked form to the relatively mild and short-lived diarrhoea generally elicited by enterotoxigenic *E.coli* is unclear. Certain wild type *V.Cholerae* strains secrete very little toxin but are still extremely virulent *in vivo*. There appears to be ample proteolytic activity in the

small bowel to activate LT. Indeed in the human or animal intestine *in vivo* gut fluid factors may be mainly responsible for both releasing LT from its periplasmic location and activating it by proteolytic nicking.

The low level of activity of LT seen in section 4.2.3 seems most likely due to inefficient or in appropriate nicking with trypsin as, despite its name, the heat labile LT is a fairly stable protein. Previous reports have shown that purified LT is equivalent to cholera toxin in specific activity in several biological assays which have been applied to both toxins (Clements and Finkelstein, 1979). The failure of LT to become photochemically labelled under conditions which resulted in efficient labelling of cholera toxin (Chapter Six) and the lack of change in the fluorescence of Nbf-LT when titrated with concentrations of NAD^+ which enhanced the fluorescence of Nbf-cholera toxin could reflect either subtle differences in the structures of the toxins or, more likely, could highlight a basic loss of activity in this particular preparation of LT.

Finally, it is worth speculating on the evolutionary origin of LT and cholera toxin and their role in the biology and ecology of their host bacteria. Man is the only host for *V.Cholerae* which survives poorly in the colon and the external environment. The toxin may confer survival advantage by increasing the numbers of bacteria released into the environment. Passage in animals reportedly favours hypertoxinogenic strains (Mekalonos *et al.*, 1983). *E.coli* is fairly ubiquitous and therefore its ability to produce LT may be of little advantage. As the toxins are as highly

homologous and seem certain to share a common origin, it seems more likely that *E.coli* received the toxin genes from *V.cholerae* rather than *vice versa*.

Chapter Five Binding of NAD^+ to cholera toxin

5.1 Introduction

In Chapter Three a K_m of 4mM for NAD^+ was calculated for the NAD^+ Glycohydrolase (NAD'ase) activity of cholera toxin. In the presence of the low molecular weight substrate analogues A599 and 4406, the apparent K_m rose even higher to around 40mM. This implies that the binding affinity of the A_1 subunit for NAD^+ may also be low and may be adversely affected by the binding of A599 or 4406.

The number of binding sites for NAD^+ on the A_1 chain has not been previously estimated. Although it may be reasonable to assume that the toxin has not evolved separate catalytic sites for the NAD'ase and ADP-ribosylation activities, this has not been proven. Poly (ADP-ribosyl) transferase enzymes isolated from the eukaryotic nucleus have been shown to contain both an initial catalytic site and one or more different acceptor sites (Bauer and Kun, 1984; Kawaichi *et al.*, 1981). For any binding interaction, the determination of stoichiometry, (the maximum number of moles of ligand bound per mole of protein, n) and of the equilibrium dissociation constant, K_d, can be done using a variety of techniques. The mathematical description of a simple equilibrium of protein, P, and ligand, A, is given by

$$P + A = PA$$

$$K_d = \frac{[P][A]}{[PA]} \qquad - 1$$

If the number of moles of A bound per mole of P is defined as r then

$$r = \frac{\text{concentration of A bound to P}}{\text{total concentration of all forms of P}}$$

$$= \frac{[PA]}{[P] + [PA]}$$

now from equation 1

$$[PA] = \frac{[P][A]}{K_d}$$

$$r = \frac{([P][A])/Kd}{[P] + ([P][A])/K_d}$$

$$r = \frac{[A]}{K_d + [A]} \qquad - 2$$

In this case r≤1 and is the fractional saturation of sites. This assumes that one mole of P can bind one mole of A.

Two basic types of experimental procedure have been used in measurements of ligand binding by proteins; so-called separation and non-separation methods. In the separation method the protein:ligand system is physically separated from free ligand (or protein) at equilibrium. This allows a direct measurement of bound ligand at equilibrium to be made without disturbing the equilibrium. Techniques include equilibrium dialysis and gel filtration. Since free ligand, A, and fractional saturation, r, are measured independently it should be possible to evaluate equilibria accurately. However these techniques can be time consuming.

The non-separation method involves correlation of some measurable

change accompanying binding with fractional saturation, r. The most commonly used techniques are spectroscopic measurements of changes in absorbance, fluorescence or optical rotary dispersion. These techniques have the advantage of both speed and sensitivity and can provide information on both K_d and the nature of the protein- ligand complex. Under certain conditions when both the exact concentration of protein is known and there is some independent way of relating ligand fluorescence to concentration, an estimate of n can also be obtained.

In this chapter a number of techniques, both separation and non-separation, have been used in an attempt to study the binding of cholera toxin and NAD^+. These included equilibrium dialysis and measurements of absorbance and fluorescence changes accompanying the binding of NAD^+. A number of experimental problems, most arising from the high concentrations of NAD^+ required to reach saturation, were encountered and are described in the Results section. Eventually an indirect method of evaluating binding based on the relationship between substrate binding and the susceptibility of the toxin's A_1 chain to protease digestion was used in order to calculate a K_d of 4mM for NAD^+. The ability of various NAD^+ derivatives to bind to the A_1 chain in place of NAD^+ was also studied using this method and the results compared with the competitive inhibitor studies described in Chapter Three.

5.2 Results

5.2.1 Equilibrium Dialysis

Equilibrium dialysis was performed using a Dianom equilibrium dialysis apparatus consisting of arrays of Teflon cells in a rotating assembly. Each cell consisted of two half cells separated by a membrane with ports in both sides for loading and unloading samples. This apparatus allows a quick and reproducible estimate of K_d and n (Weder *et al.*, 1971). A number of preliminary experiments were performed to characterise the system in terms of equilibrium time, stability of ligand and protein etc. and these are described below.

5.2.1.1 Dialysis time

The time taken to reach equilibrium under the experimental conditions outlined in section 2.11 was determined by the time taken for solutions of [4-^{3}H] NAD^+ (20nCi) containing 0.1 - 10mM unlabelled NAD^+ to equilibrate between two half cells of the dialysis equipment. Samples were taken through ports from both sides of the membrane over an incubation time of four hours at 37°C and the radioactivity counted. Equilibrium was reached after 120 min.

5.2.1.2 Recovery of radioactivity

When [4-^{3}H] NAD^+ was dialysed against cholera toxin A_1 chain (0.1 mg/ml) comparison of the total amount of radioactivity added with the amounts found in each half cell during dialysis showed that more than 95% of the total radioactivity was recovered. The small loss of radioactivity was most probably caused by the ligand binding to the dialysis membrane.

5.2.1.3 Stability of protein and ligand

To check that the cholera toxin used retained biological activity after dialysis, samples of the A_1 chain or cholera toxin were dialysed against either 0.1M borate, pH8.5 or TEAN buffer, pH7.5. Following incubation at 37°C for up to four hours, 50µl samples were removed from the protein-containing half cell and were assayed by the ADP-ribosylation assay described in section 2.9. Samples were also analysed on 12% SDS-PAGE in the presence of thiol to see whether aggregation or degradation had occurred. In all cases the dialysed samples retained 80 - 100% of their original activity and their apparent molecular weight was unaffected. To determine the integrity of the NAD^+ after dialysis against cholera toxin, samples were removed at the end of each experiment and were analysed by tlc as described in section 2.10. In all cases breakdown of NAD^+ to nicotinamide was below 5%. This indicates that unlike diphtheria toxin which can completely hydrolyse a ten fold molar excess of NAD^+ within two hours (Kandel *et al.*, 1974), the NAD^+ Glycohydrolase activity of cholera toxin should not complicate the dialysis experiments.

5.2.1.4 Binding experiments

Preliminary experiments were attempted using 1µM (0.1 mg/ml) cholera toxin and 0.02 - 2mM NAD^+ containing 20nCi [$4^{-3}H$] NAD^+. The results failed to detect any significant binding of NAD^+. With results from kinetic experiments suggesting values of K_m for NAD^+ of 4mM in the absence of acceptors, it seemed that much higher concentrations of both NAD^+ and protein would be required. If the probability of binding, p, is defined as

$$p = \frac{\text{actual measurement of protein:ligand complex}}{\text{maximum possible concentration of protein:ligand complex}}$$

then for stoichiometric measurements p should ideally equal one. Protein concentration should therefore be as high as possible so that as much ligand as possible is bound but not so high as to cause problems with protein solubility and the Gibbs Donnan effect (van Holde, 1971) which can upset the equilibrium. Experiments were repeated using 12μM (1 mg/ml) cholera toxin in TEAN buffer pH7.5 containing 5mM DTT and using 0.01 - 5.0 mM NAD^+. The data were first analysed using a computer programme based on the method of Marquardt (1963) (Appendix A). The results are also presented graphically as a plot of 1/r vs 1/A (fig. 5-1), a double reciprocal plot of mol ligand bound per mol. protein versus free ligand concentration (Hughes-Klotz plot). This plot was used in preference to the more familiar Scatchard plot (Scatchard, 1949). Nimmo *et al.*, (1977) reviewed a number of methods of analysing data obtained from experiments of similar design to those described here, and concluded that the regression of 1/r on 1/A was the best because it gave estimates that were accurate, precise and symmetrically distributed. This regression is loosely equivalent to the Lineweaver-Burk double-reciprocal plot of enzyme kinetics (Lineweaver and Burk, 1934) which is not favoured by many workers because of the unequal distribution of errors it produces (Price and Dwek, 1979).

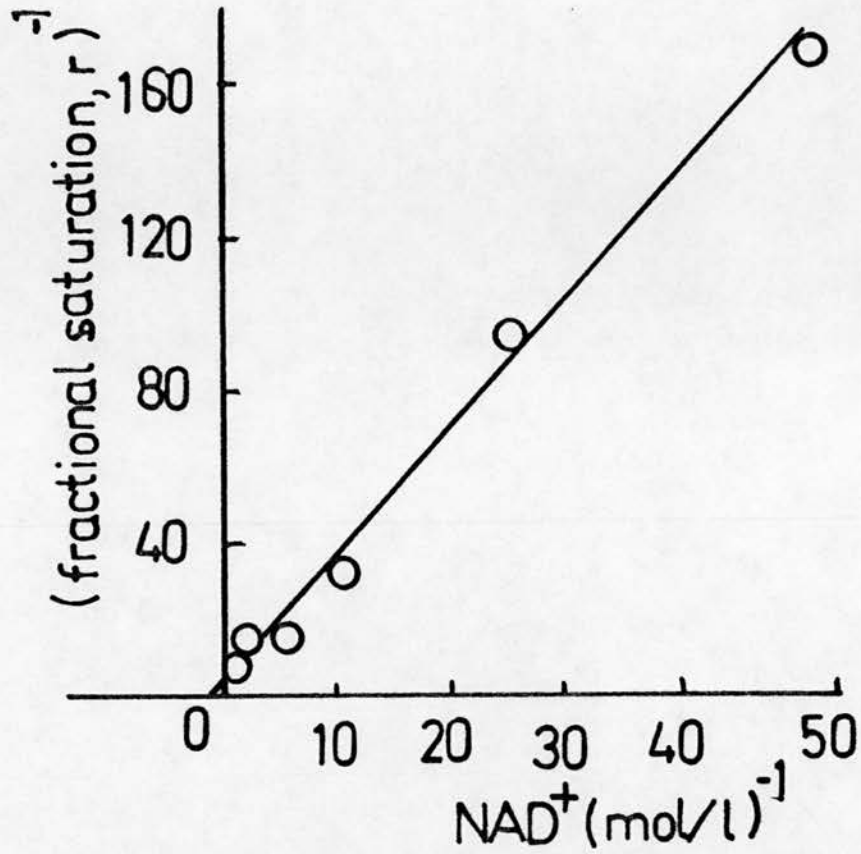


fig. 5-1 Double reciprocal plot of ligand binding data

Cholera toxin (12μM) was dialysed against concentrations of NAD^+ between 0.01 and 5.0mM.
Results were analysed by computer (Appendix A) and are presented here as a plot of 1/r versus 1/A, where r is calculated from b (bound ligand in mol l^{-1})

Presumably their differing suitabilities arise because in enzyme kinetics one of the variables (substrate concentration) is genuinely independent in the statistical sense (i.e. fixed by the observer and effectively free from error) whereas in equilibrium dialysis both of the derived variables, r and A are subject to experimental error which differs for each ligand concentration.

The binding parameters calculated from these data were $n = 1.0 \pm 0.8$ and $k_d = 3.0 \pm 2.8$mM. The error involved in these calculations is considerable and makes it doubtful whether the values, especially of K_d can be considered accurate. These errors may be due in part to the high concentrations of NAD^+ being used (which mean that the concentration of bound ligand accounts for a very small difference between two large numbers). For example at the lowest concentration of ligand, 0.1 mM NAD^+, an experimental error in estimating free ligand radioactivity of 1% could lead to errors in r of about 4%. At the highest concentration of ligand (5mM) the comparable value would be up to 20% error in the estimate of r.

Binding of NAD^+ to the A_1 chain alone was also studied. Using conditions as described for cholera toxin and with concentrations of A_1 chain up to 10μM (0.2 mg/ml) no binding of ligand could be detected. Increasing the concentration of NAD^+ and of A_1 chain proved difficult as at the concentrations of protein required (up to 2 mg/ml or 80μM) the A_1 chain came out of solution after a few minutes. Although 2.5M urea could effectively keep the A_1 chain in solution alternative methods were sought as the urea, being a chaotropic agent, could have been disrupting the native conformation

of the protein so as to interfere with the binding of its substrate. Substitution of 0.1M borate, pH8.5 for TEAN buffer allowed the A_1 concentration to be increased to 2 mg/ml. However, when these concentrations of A_1 were used in binding experiments, the data obtained showed errors of up to 25% in the quadruplet samples cpm values. It appeared that during the course of the experiments (up to two hours) the A_1 chain was gradually precipitating. Despite numerous attempts, no results suitable for analysis were obtained using the A_1 chain.

5.2.2 Quenching of protein fluorescence by NAD^+

For both diphtheria toxin fragment A (Kandel *et al.*, 1974) and an active peptide derived from pseudomonas exotoxin A (Chung and Collier, 1977b) the binding of NAD^+ causes a quenching of the intrinsic fluorescence (excitation 285nm, emmission 333nm) of the toxins. The intrinsic fluorescence of diphtheria toxin fragment A was reduced by up to 60% while that of the exotoxin A peptide was reduced by about 20%. In both cases the concentration of NAD^+ that caused half maximal quenching was about 9μM, which correlated well with the K_m of 8μM for NAD^+ for their ADP-ribosylation activities. The quenching is thought to be the result of a charge transfer complex between the nicotinamide moiety of NAD^+ and a tryptophan residue within each protein.

To determine whether a similar effect was evident for cholera toxin or its A_1 chain alone, the intensity of fluorescence of cholera toxin (1mg/ml, 12μM) or A_1 chain (0.5mg/ml, 20μM) in either TEAN buffer, pH7.5, phosphate buffer, pH6.5 or borate buffer, pH8.5 was measured in the presence of increasing concentrations of NAD^+ as

described in section 2.13. As shown in fig. 5-2 at saturating concentrations of NAD^+ (4mM and above) the fluorescence at 333nm (excitation 285nm) of whole toxin, at pH7.5, was reduced to a level of about 50% of its initial value. A similar effect was seen for the A_1 chain. Although this at first appeared to represent a direct effect of NAD^+ on the intrinsic fluorescence of cholera toxin, the decrease in fluorescence could be explained fully by the increased absorption of NAD^+ at 285nm.

Changing the excitation wavelength to 290nm at which wavelength the absorbance of NAD^+ is significantly lower, was not enough to prevent the absorption of NAD^+ from reducing the effective intensity of light reaching the sample as shown in table 5-1. This in turn reduced the intensity of fluorescence.

mM NAD^+	A_{290}nm	proportion of light transmitted
0	0	1.0
0.1	0.053	0.94
0.5	0.265	0.73
1.0	0.53	0.54
2.0	1.06	0.30
5.0	2.65	0.05
10	10.6	5×10^{-6}

Table 5-1 The contribution of NAD^+ absorbance at 290nm to the proportion of light transmitted to the sample

The absorbance of a 1mM solution of NAD^+ at 290nm was taken to be 0.53

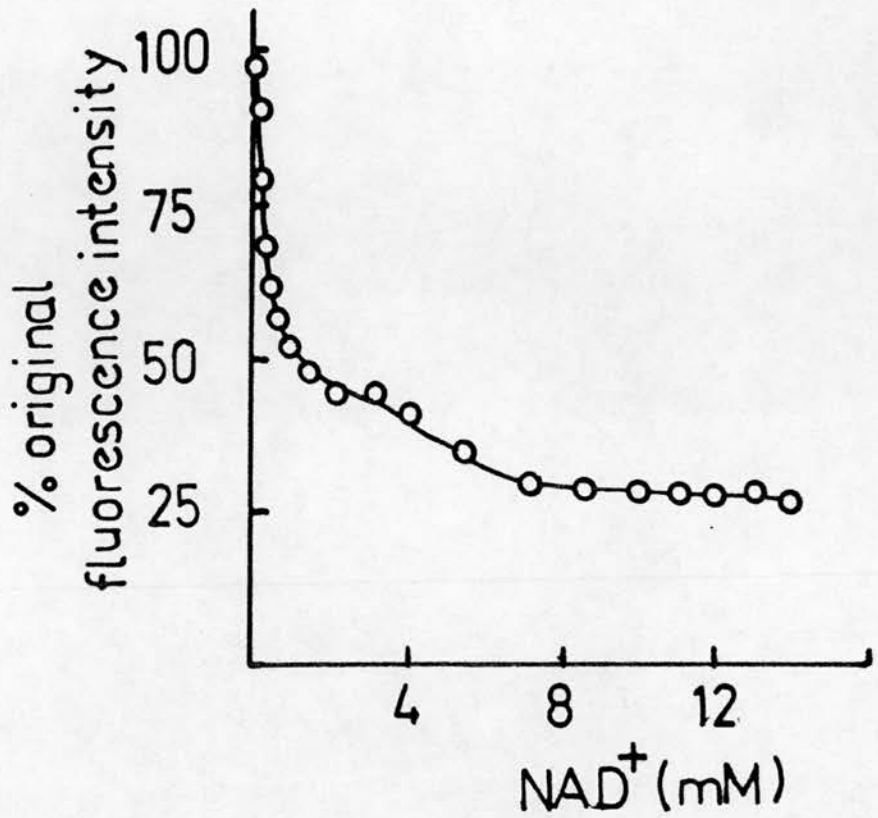


fig. 5-2 Decrease in fluorescence intensity as a function of NAD^+ concentration

The decrease in fluorescence intensity (ex290/em330) was recorded when NAD^+ was added to cholera toxin, 12µM in TEAN buffer, pH7.5 at 25°C.

The absorbance of NAD^+ was not a problem when diphtheria toxin and exotoxin A were examined at this wavelength as the concentration of NAD^+ required to reach saturation was a thousand fold lower. It is impossible to say from these results whether or not NAD^+ causes a change in the intrinsic fluorescence of cholera toxin as any effect at this wavelength is effectively swamped by the absorbance of NAD^+.

5.2.3 Ultra violet difference spectra

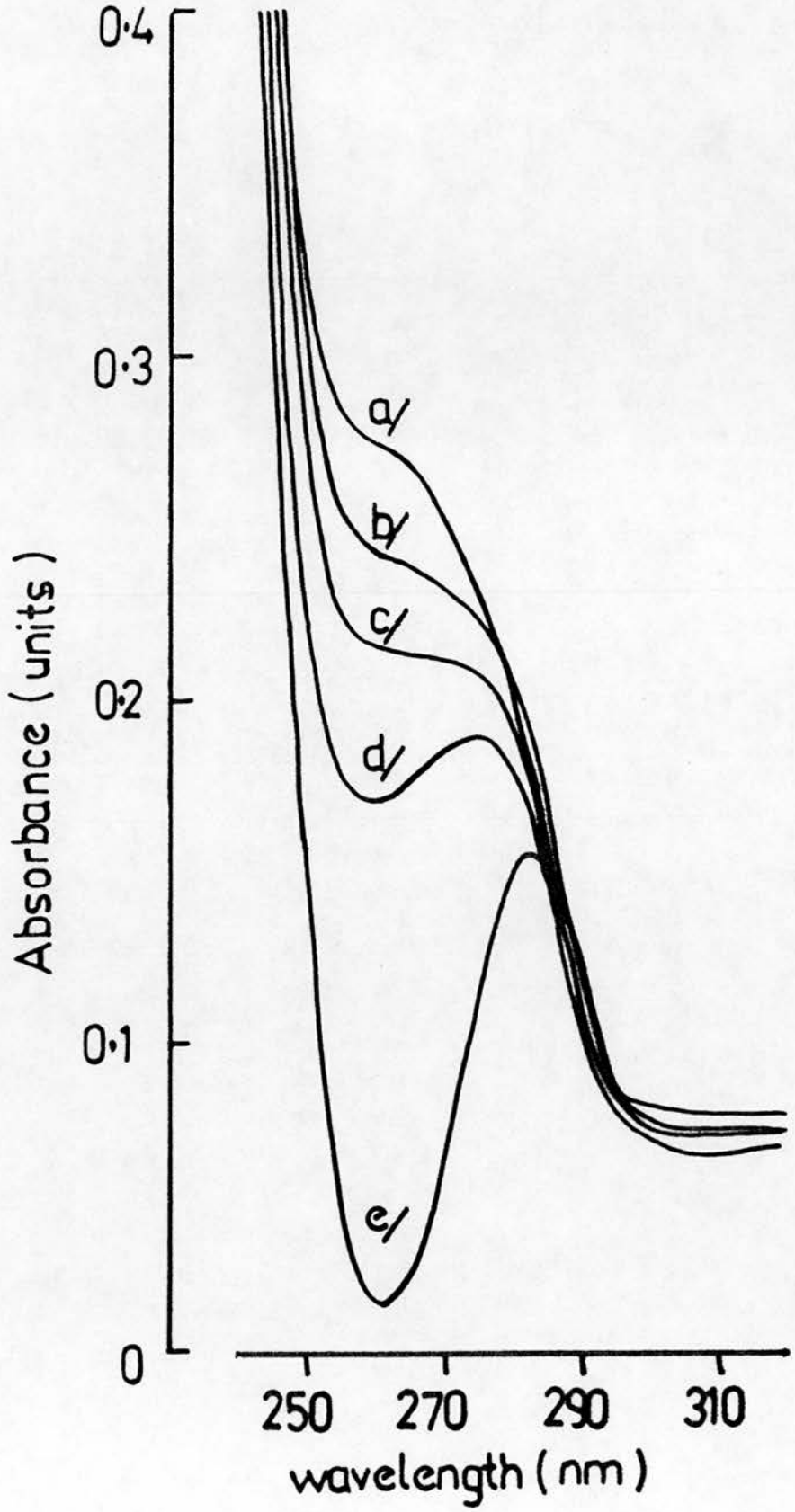
When NAD^+ binds to diphtheria toxin fragment A, it causes alterations in the fragment's spectral properties. Difference spectra revealed a red shift in the absorbance peaks at 281nm for tyrosine and 294nm for tryptophan presumably due to transfer of these residues into a more hydrophobic environment. There was also a new absorbance band produced at 360nm. When plotted as a function of NAD^+ concentration the absorbance at 360nm lay on a theoretical curve calculated from the binding of NAD^+ with fragment A of 8μM (Kandel *et al.*, 1974).

The difference spectrum of the A_1 chain generated by NAD^+ was recorded using pairs of matching tandem double quartz cuvettes (1cm light path). NAD^+ solution was added in microlitre quantities to a 15μM solution of the A_1 chain in TEAN buffer at pH7.5 and to buffer in the reference cuvette. An equal volume of buffer was added to the protein reference cuvette. Difference spectra were recorded using a Pye Unicam SP800 spectrophotometer. The temperature in the water-jacketed cuvette chamber was kept at 25°C. Results are presented in fig. 5-3 of the change in absorbance seen at 269nm. No change in absorbance was seen at 360nm. The results are difficult to interpret because of the strong absorbance of NAD^+ itself at

260nm. How much of the apparent quench was due to an actual change in the A_1 chain's spectral properties and how much due to difficulties in matching the sample and reference cuvettes after the addition of NAD^+ is not apparent.

fig. 5-3 Difference spectra of cholera toxin A_1 chain generated by NAD^+

To cholera toxin A_1 chain 15μM in TEAN buffer and to the buffer in the reference cuvette NAD^+ was added in 300μM increments. (a = no NAD^+, b = 300μM, c = 600μM, d = 900μM, e = 1200μM NAD^+)


0·4
0·3
0·2
0·1
0
Absorbance (units)
a/
b/
c/
d/
e/
250
270
290
310
wavelength (nm)

5.2.4 Cholera toxin labelled with 7-chloro-4-nitrobenzofuran (Nbf)

As there was no measurable change in the intrinsic fluorescence of cholera toxin following the binding of NAD^+, fluorescently labelled toxin was used instead to try to detect some measurable change following the binding of NAD^+. Portions of cholera toxin, its A_1 chain and *E.coli* heat labile toxin (LT, see Chapter Four and in particular section 4.2.3) were labelled with the fluorescent probe Nbf. Nbf reacts most readily with free sulphydryl groups and has generally been used in this way. There are no free sulphydryl groups in cholera toxin but at pH8.5 Nbf will also react with the amino groups of lysine residues of which there are several in both subunits of the toxin (Duffy *et al.*, 1985; Lai, 1977).

5.2.4.1 Preparation of Nbf-labelled toxin and subunits

A solution of cholera toxin or its A_1 chain alone was labelled with Nbf by incubating with a near saturating solution at pH8.5 as described in section 2.13. After exhaustive dialysis, the product showed the properties expected of the Nbf derivative of a protein with an absorbance maximum at 475nm and a fluorescence maximum at about 530nm with the excitation maximum at 468nm. When the labelled toxin was analysed by 12% SDS-PAGE it showed three bands (or one band for labelled A_1 chain) visible under ultra violet light and staining with coomassie blue. These bands had the expected mobility of subunits A_1, A_2 and B.

A portion of the labelled toxin was separated into its constituent subunits by gel filtration chromatography in 6.5M urea at pH3.2 (section 2.3). Urea was removed from the solution by dialysis as described. The B subunit was collected and shown to be

pure on 12% SDS-PAGE. Measurement of the absorbance for protein at 280nm and for the amino derivative of Nbf at 475nm suggested that about 2 moles of Nbf had bound per mole of cholera toxin, 2 moles per mole of the A_1 chain and 1 mole per mole of the purified B_5 pentamer. After labelling and separation, cholera toxin A_1 chain retained its biological activity in the ADP-ribosylation assay described in section 2.9, while subunit B retained the ability to bind ganglioside as judged by diffusion experiments described in section 2.3.

5.2.4.2 Fluorescence of the Nbf-toxin and the effect of NAD^+

When a solution of Nbf-labelled toxin was treated with NAD^+ as described in section 2.14 there was a marked increase in the fluorescence intensity but no measurable change in the wavelength of maximum intensity which stayed at 533nm. As shown in fig. 5-4, the addition of 150μM NAD^+ resulted in an increase in fluorescence from 3.9 arbitrary units to 7.5 arbitrary units, an increase of over 50%. The result of a quantitative titration of a solution of Nbf toxin (15μM) in TEAN buffer, pH7.5 with NAD^+ is shown in fig. 5-5. The effect was saturable, the fluorescence reaching a maximum after 160μM NAD^+ had been added. There was, however, a lag at the start of the titration curve, with no enhancement of fluorescence evident until 25μM NAD^+ had been added. Similar enhancement was seen when Nbf-A_1 chain was titrated with NAD^+. No change was seen in the fluorescence of Nbf-B subunit on addition of NAD^+. The addition of saturating concentrations of the arginine derivative A599 (see fig. 3-1) produced no change in the fluorescence of Nbf-labelled cholera toxin, A_1 chain or B subunit, either in the presence of absence of

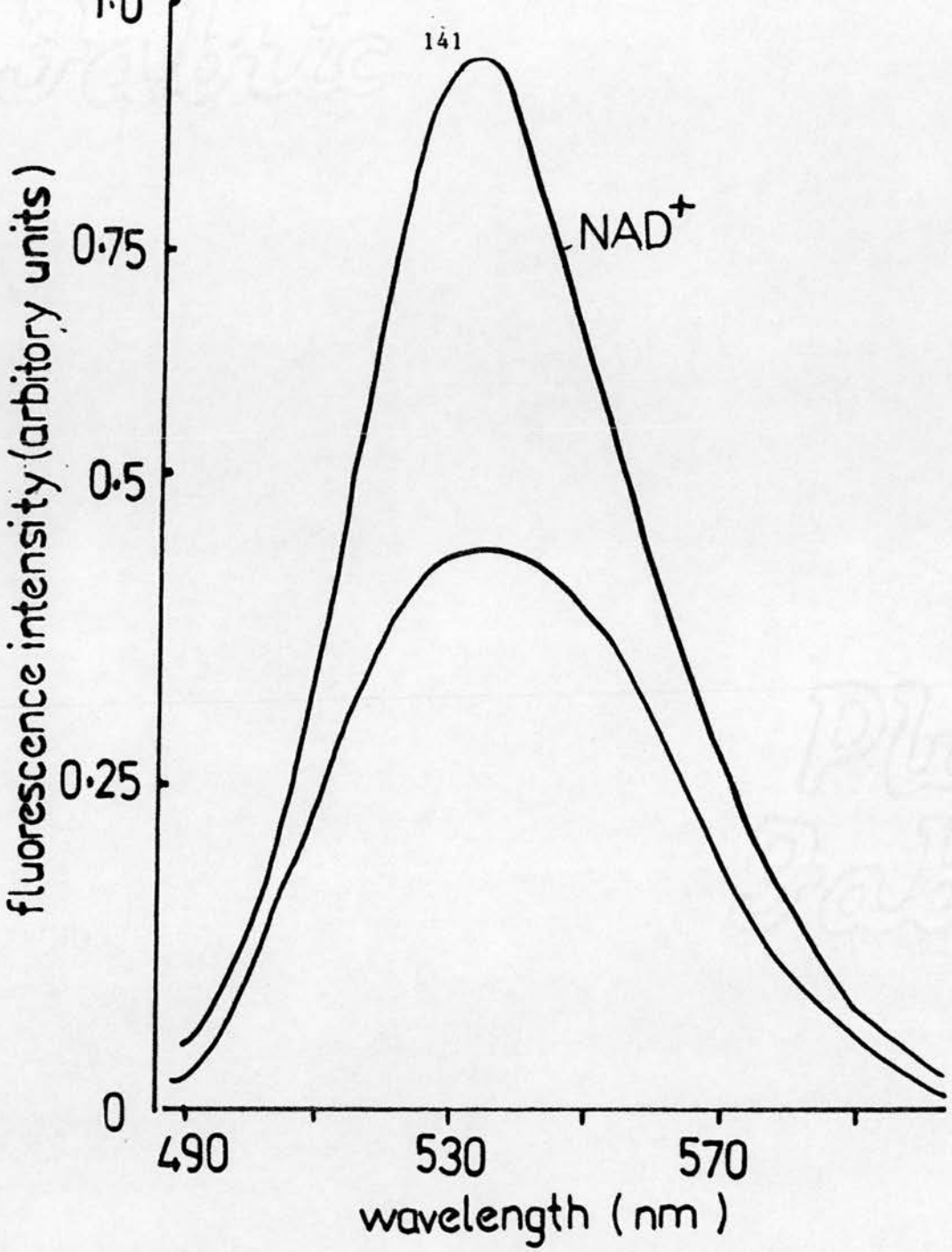


fig. 5-4 **Fluorescence intensity of Nbf-toxin in the presence and absence of NAD⁺**

The toxin was approximately 10μM in TEAN buffer, pH7.5 at 25°C. The excitation wavelength was 468nm and the excitation and emission slits were 15nm and 5nm respectively.

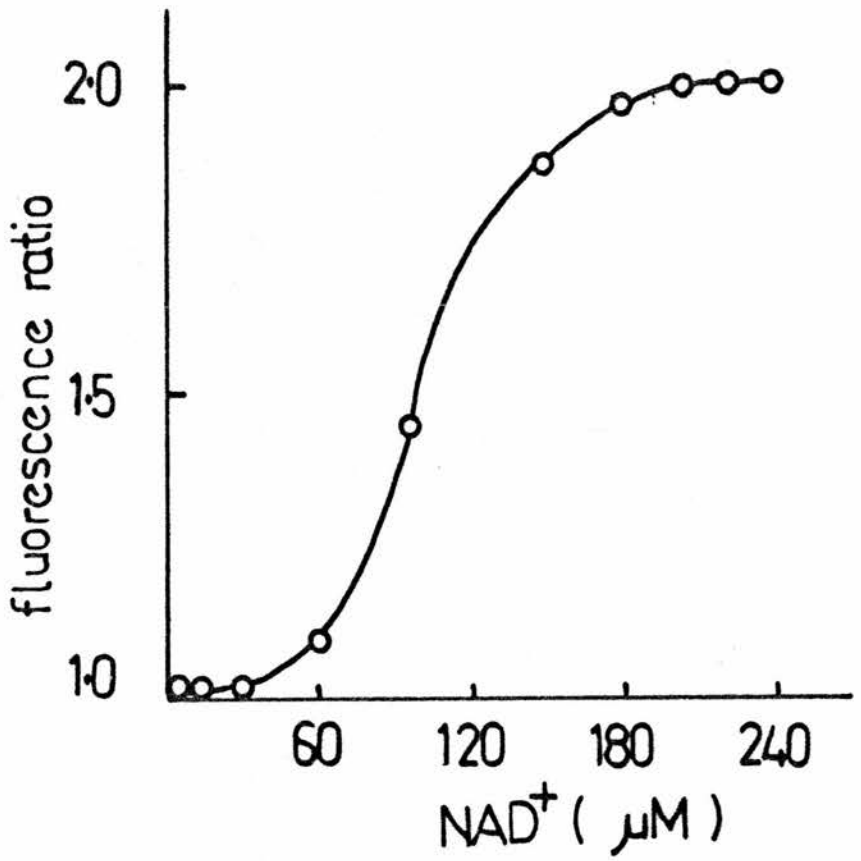


fig. 5-5 Fluorescence titration of Nbf-toxin

The toxin was approximately 15μM. Conditions are as in fig. 5-4. The ordinate is the ratio of the fluorescence intensity of the toxin solution to that of a solution of Nbf-myoglobin of comparable initial fluorescence intensity. Note the lag seen at low concentrations of NAD^+.

NAD^+

In equilibrium dialysis, separation of bound and free ligand at equilibrium allowed a direct measurement of free ligand, free protein and protein:ligand complex. From these values a calculation of r, the fractional saturation was made. Alternatively, an indirect estimate of r can be made based on some measurable change in either protein or ligand which is assumed to accompany protein: ligand binding. The bound and free ligand do not then need to be separated. Here the assumption made is that the enhancement of fluorescence at 533nm can be correlated to r. More specifically, the ratio of the fluorescence intensity of a solution of Nbf-toxin to that of a solution of Nbf-toxin fully saturated with NAD^+ is interpreted as correlating with fractional saturation. This assumes that only the protein contributes towards the fluorescence and that the ligand, NAD^+, neither absorbs nor fluoresces. Returning to equation 2 in the introduction to this Chapter,

$$r = \frac{[A]}{K_d + [A]}$$

rearrangement gives

$$\frac{1}{r} = 1 + \frac{Kd}{[A]}$$

A plot of 1/r versus 1/[A] should give a straight line of slope K_d. The change in relative fluorescence intensity can be substituted for r as follows.

$$\frac{1}{f} = \frac{1}{f_{max}} + \frac{Kd}{[A]}$$

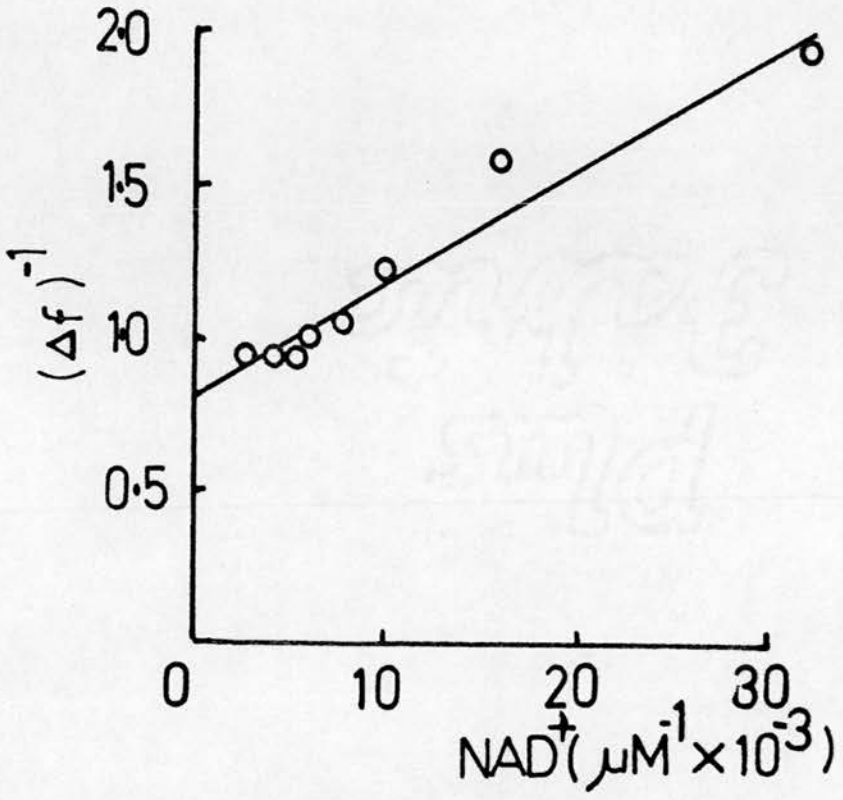


fig. 5-6 Double reciprocal plot of fluorescence titration results

The data presented in fig. 5-5 are plotted here as $1/\Delta f$ (Δf = the ratio of the fluorescence intensity of a solution of Nbf-toxin to that of a solution of Nbf-toxin fully saturated with NAD^+) versus $1/NAD^+$.
A value of K_d = 40±4μM for NAD^+ was tentatively calculated from this graph.

where f_{max} is the maximum change in relative fluorescence intensity when the system is saturated with NAD^+ and f is the change in relative fluorescence intensity at a particular concentration of NAD^+. A plot of 1/ f versus 1/[A] for the data in fig. 5-5 is shown in fig. 5-6. The estimated K_d value from the slope of this plot was $K_d = 40 \pm 4\mu M$. This value was lower than might be expected bearing in mind the millimolar K_m for NAD^+ calculated in Chapter Three and the millimolar estimate of K_d obtained using equilibrium dialysis. The lag at the start of the titration curve in fig. 5-6 cannot be explained by this model. In fact, although the curve indicates that saturation with NAD^+ occurs at around 200μM NAD^+ the curve is not a typical saturation curve at all, a point which argues against the subsequent analysis of the data in this way. The assumptions made concerning fluorescence enhancement and fractional saturation may be incorrect and an estimate of K_d based on this data would then be invalid. Whether or not the estimate of a micromolar K_d value is correct, the results indicate at least that NAD^+ is capable of causing a change in conformation of cholera toxin or of its A_1 chain alone, but not of its B subunit, and that the concentration of NAD^+ necessary to bring about this change is approximately one hundred fold lower than the estimated K_m value for NAD^+.* Why such an effect should occur at these NAD^+ concentrations is not clear. The ability of NAD^+ to bind to the A_1 chain may have allowed it to interact with the Nbf probe in a way that is not directly proportional to the amount of NAD^+ bound. As already mentioned, NAD^+ had no effect on the fluorescence of Nbf-proteins to which it does not bind (such as Nbf-B subunit and Nbf-myoglobin).

* the K_m of 3mM calculated in chapter 3 and the K_D of 4mM obtained in section 5.2.5.2.

An alternative explanation for the K_d value could be that potential experimental errors are too great to allow an accurate measurement. Weber (1965) presented a mathematical analysis of this problem based on the concept of probability of binding, p, which is defined as:

$$p = \frac{\text{actual concentration of [PA] complex}}{\text{maximum possible concentration of [PA] complex}}$$

when $[A] \geq [P]$

$$p = \frac{[PA]}{[P]-r}$$

when $[A] \leq [P]$

$$p = \frac{[PA][A]}{[A]}$$

$$= \frac{\frac{[P]}{K_d}(1-r)}{\frac{[P]}{K_d}(1-r) + 1}$$

The results of Weber's analysis relate error in K_d to the probability of binding. Error in K_d is a minimum when $p = 0.5$ whereas it increases sharply in the range above 0.8 and below 0.2 . A protein:ligand equilibrium can be accurately determined only when the protein concentration is comparable in magnitude or smaller than K_d according to this analysis. The protein concentration, at 15μM, is below the estimated K_d value here so an accurate estimate should be feasible.

5.2.5 The relationship between the susceptibility of the A_1 chain to digestion and substrate binding.

The techniques used so far have either involved making direct measurements of NAD^+ concentration as in equilibrium dialysis or have involved indirect estimates of binding based on spectroscopic or fluorescence changes. In equilibrium dialysis, the errors involved in measuring a tiny amount of bound NAD^+ in the presence of a large amount of unbound ligand led to a varied and inaccurate estimate of K_d. In intrinsic fluorescence and difference spectrum measurements the absorbance at 260nm of the large concentrations of NAD^+ necessary to reach saturation swamped any changes in the fluorescence or absorbance of toxin. The use of Nbf-labelled toxin avoided the problem of NAD's strong absorbance but yielded a K_d value (K_d = 40±15μM) which did not seem to agree with the concentrations of NAD^+ necessary for catalytic activity (see Chapter Three) and was in other ways unsatisfactory. An alternative method of calculating a K_d value was sought.

It was first noted by Karen Harper, a summer student working with Dr. Simon van Heyningen in the Department of Biochemistry, University of Edinburgh, that the A_1 chain of cholera toxin produced a clear and consistent pattern of bands when digested with trypsin and analysed by 15% SDS-PAGE. Of most interest was the subsequent finding that 50mM NAD^+ could completely protect the A_1 chain from digestion (fig. 5-7). This was taken as evidence that the NAD^+ was inducing a conformational change in the A_1 chain upon binding that drastically altered its susceptibility to protease digestion. It was pointed out by Dr. David Apps that a method based on the concept

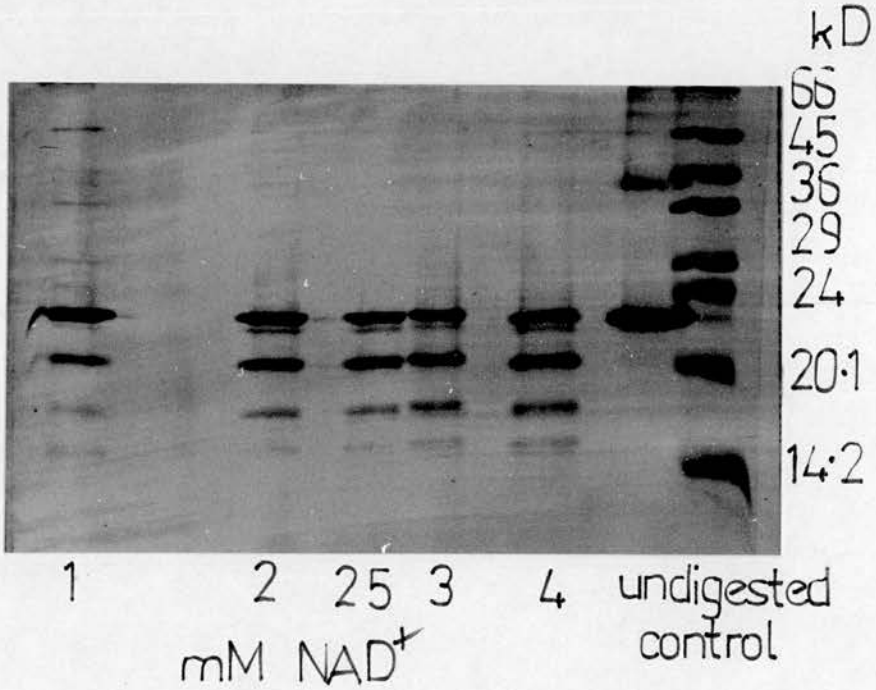


fig. 5-7 15% SDS-PAGE showing cholera toxin A_1 chain treated with 5µg/ml trypsin in the presence of 0-4mM NAD^+

of using changes in the rate of tryptic digestion as a measure of substrate binding had been used successfully by Jacobs and Cunningham (1968) to calculate binding constants for the interaction of creatine kinase with its substrates. Their method relied on an indirect estimate of substrate binding measured by the ability of each substrate to protect the protein from digestion. Jacobs and Cunningham used a pH stat meter to measure the rate of digestion of creatine kinase. Here the initial rate of digestion of cholera toxin's A_1 chain was measured by the appearance of digestion bands on 15% SDS-PAGE using a densitometric scanner as described in sections 2.16 and 2.7.3.

5.2.5.1 Protection of the A_1 chain from proteolysis

Samples of the A_1 chain in TEAN buffer pH7.5 containing a total of 25µg protein were incubated with trypsin for periods of up to an hour. The reaction was stopped by the addition of 1mM PMSF in propan-1-ol and the digested protein was analysed by 15% SDS-PAGE. A clear pattern of bands appeared ranging in molecular weight from approximately 20,000 to 12,000. The appearance of the largest band of molecular weight 20,000 was measured in each case. The appearance of this band rather than the disappearance of the original A_1 band (molecular weight 22,000) was measured as its appearance represented an easily measurable increase rather than a small gradual decrease in a denser protein band as was the case for the larger band. However, results from the disappearance of the 22,000 band or from an average of the appearance of the bands at 20,000, 16,000 and 14,000 gave essentially the same value for the rate of digestion over 30 min.

To determine the optimum conditions for digestion, samples of the A_1 chain were incubated with 1-50µg/ml trypsin for times up to one hour. The clearest pattern of digested bands was obtained using 5µg/ml trypsin with digestion continued for up to 30 min. Whole cholera toxin was not used for these experiments as the pattern of digestion bands became too complicated.

A time course for the digestion of the A_1 chain by trypsin is shown in fig. 5-8(a). Fig. 5-8(b) shows a densitometric scan of one lane of this gel. When the appearance of the 20,000 band was plotted as a function of time, a rapid initial burst of digestion was seen followed by a much slower reaction which exhibited a relatively constant rate for at least 20 min. (fig. 5-9). It is the slope of the linear portion of this graph which has been used to compare the susceptibility of the protein to tryptic digestion under various conditions. The reason for the initial rapid burst of digestion is not certain. It could be caused by a rapid digestion of susceptible surface or denatured bonds followed by a slower but relatively constant rate of digestion. Alternatively the linear portion could represent a steady state as the 20,000 band is formed and degraded. When digestion was allowed to proceed beyond 30 min., the 20,000 band gradually disappeared as it was digested to form smaller peptides. Whatever the complex kinetic explanation behind the biphasic digestion rate, the linear portion is clearly a function of, and is related to, the NAD^+ concentration and was measured in each case.

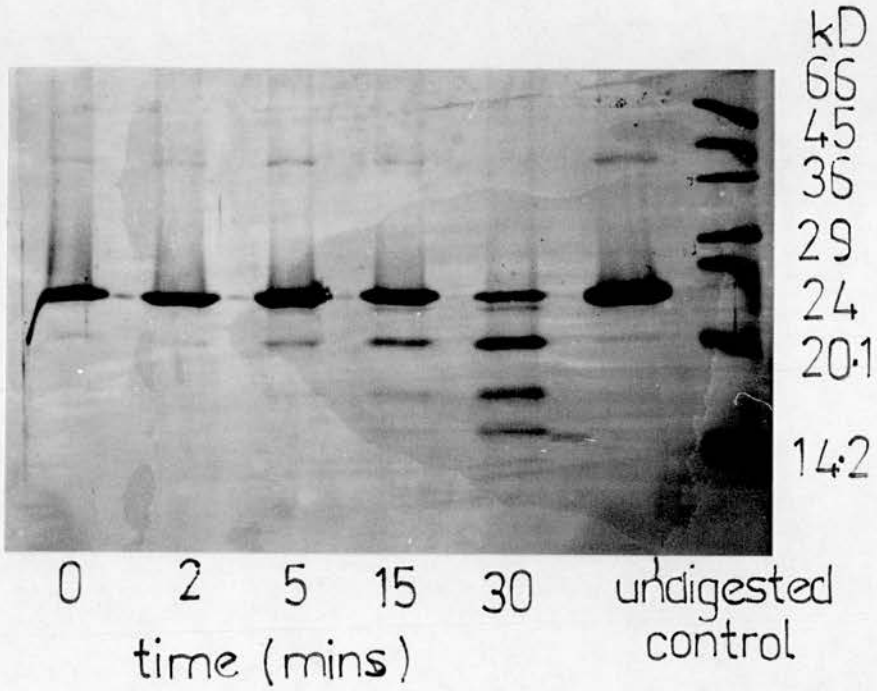


fig. 5-8(a) **15% SDS-PAGE showing the rate of digestion of the A_1 chain by trypsin.**

Conditions were as outlined in the text

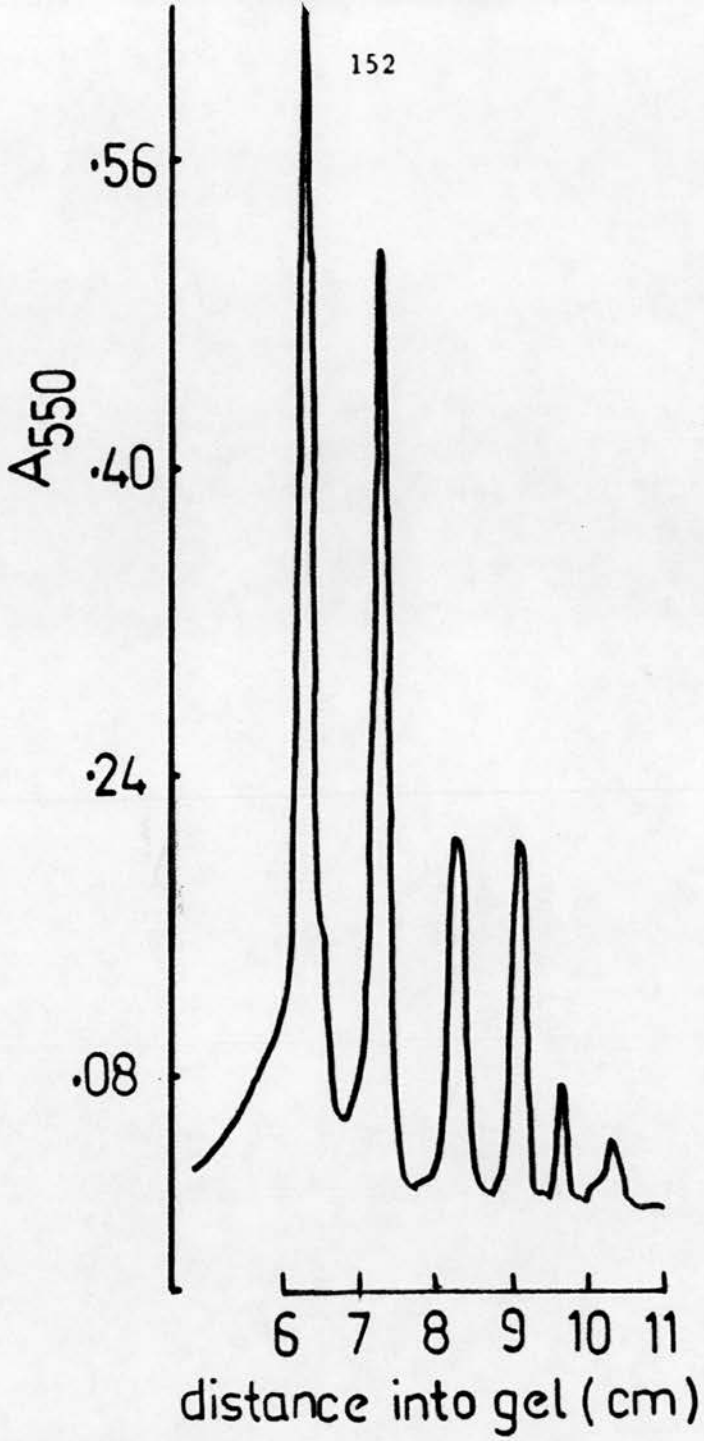


fig. 5-8(b) Desitometric analysis of a tryptic digest of the A_1 chain (25µg/ml)

Trypsin was added to a concentration of 5µg/ml and digestion allowed to proceed for 30 min at 37°C

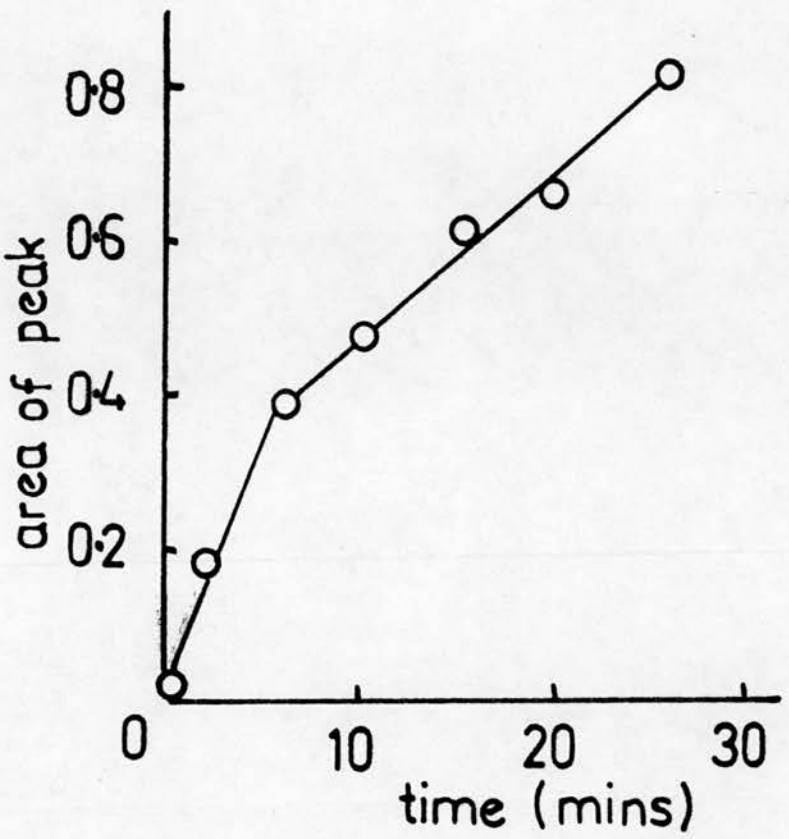


fig. 5-9 Rate of digestion of the A_1 chain by trypsin

The rate of digestion was measured by the appearance of a digestion band of molecular weight (approximately) 20,000

In experiments to determine the extent of substrate protection by NAD^+, concentrations of NAD^+ of up to 100mM were added to the A_1 chain and left to stand for 10 min at room temperature prior to digestion. The presence of 50mM NAD^+ was found to inhibit digestion of the A_1 chain completely for up to one hour at 37°C. NAD^+ also protected the A_1 chain from digestion by chymotrypsin (5μg/ml) but to a lesser extent, with some digested bands apparent after a few minutes even with 50mM NAD^+. The more complex pattern of bands seen following digestion with chymotrypsin and the less easily interpreted densitometric scan are shown in fig. 5-10.

When the A_1 chain was digested with trypsin in the presence of 10mM A603 (one of the arginine derivatives described in Chapter Three and illustrated in fig. 3-1) there was essentially no digestion over 30 min. (fig. 5-11). Digestion was inhibited by concentrations of A603 down to 0.5mM (the lowest concentration tested, but not necessarily the lowest concentration to cause an effect). This substrate protection is interpreted to be caused by a conformational change in the A_1 chain brought about by the binding of A603. This shows that the substrate analogue A603 can bind to the toxin's A_1 chain in the absence of NAD^+. Analysis of the data obtained allowed only a rough estimate of the K_d for A603 which was around millimolar.

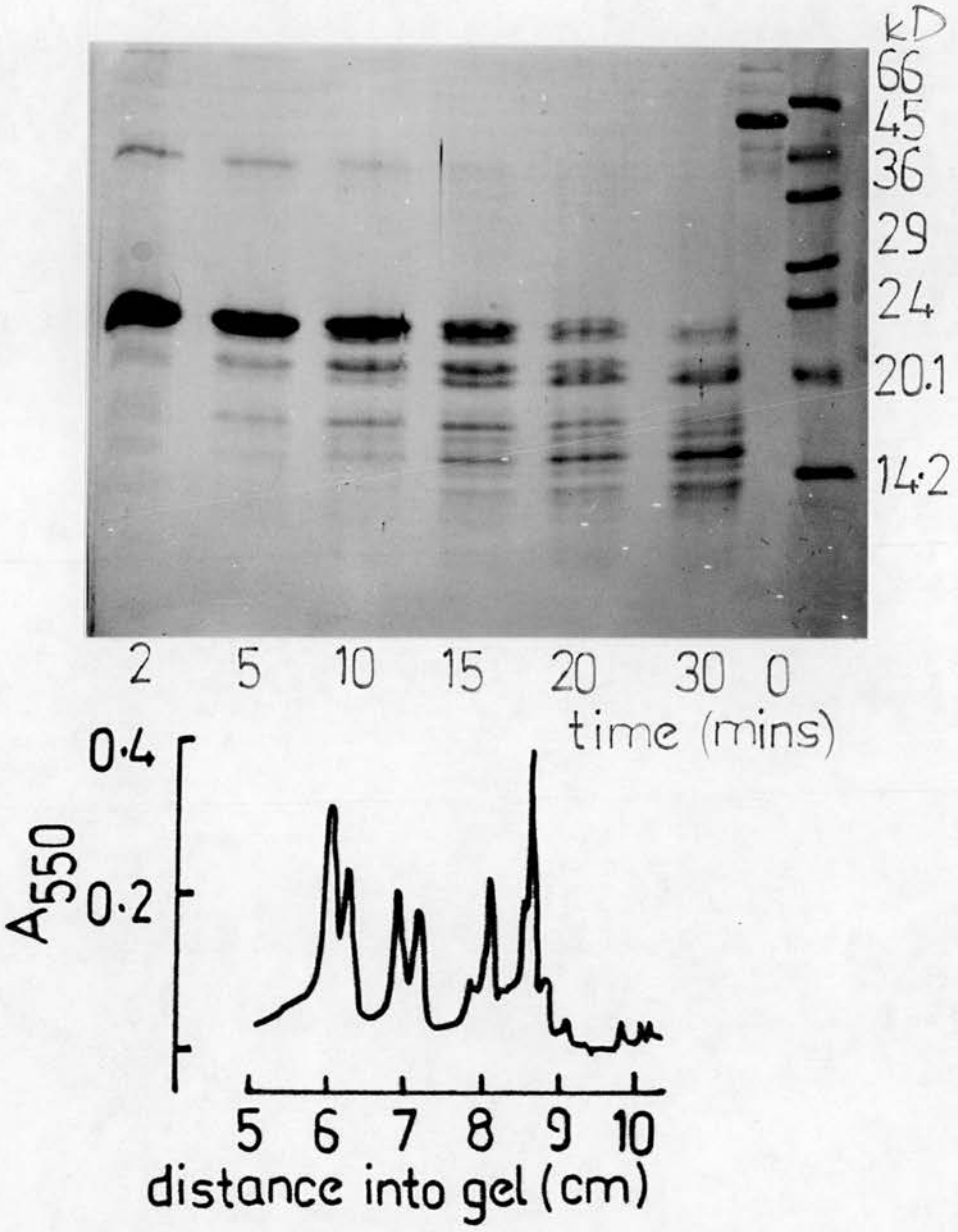


fig. 5-10 Digestion of the A_1 chain with chymotrypsin

A_1 chain (25µg) was digested with 5µg/ml chymotrypsin for 30 min. at 37°C as described. The photo shows the rate of digestion by chymotrypsin in the presence of 10mM NAD^+. The densitometric scan is of a sample of A_1 chain digested with chymotrypsin for 20 min.

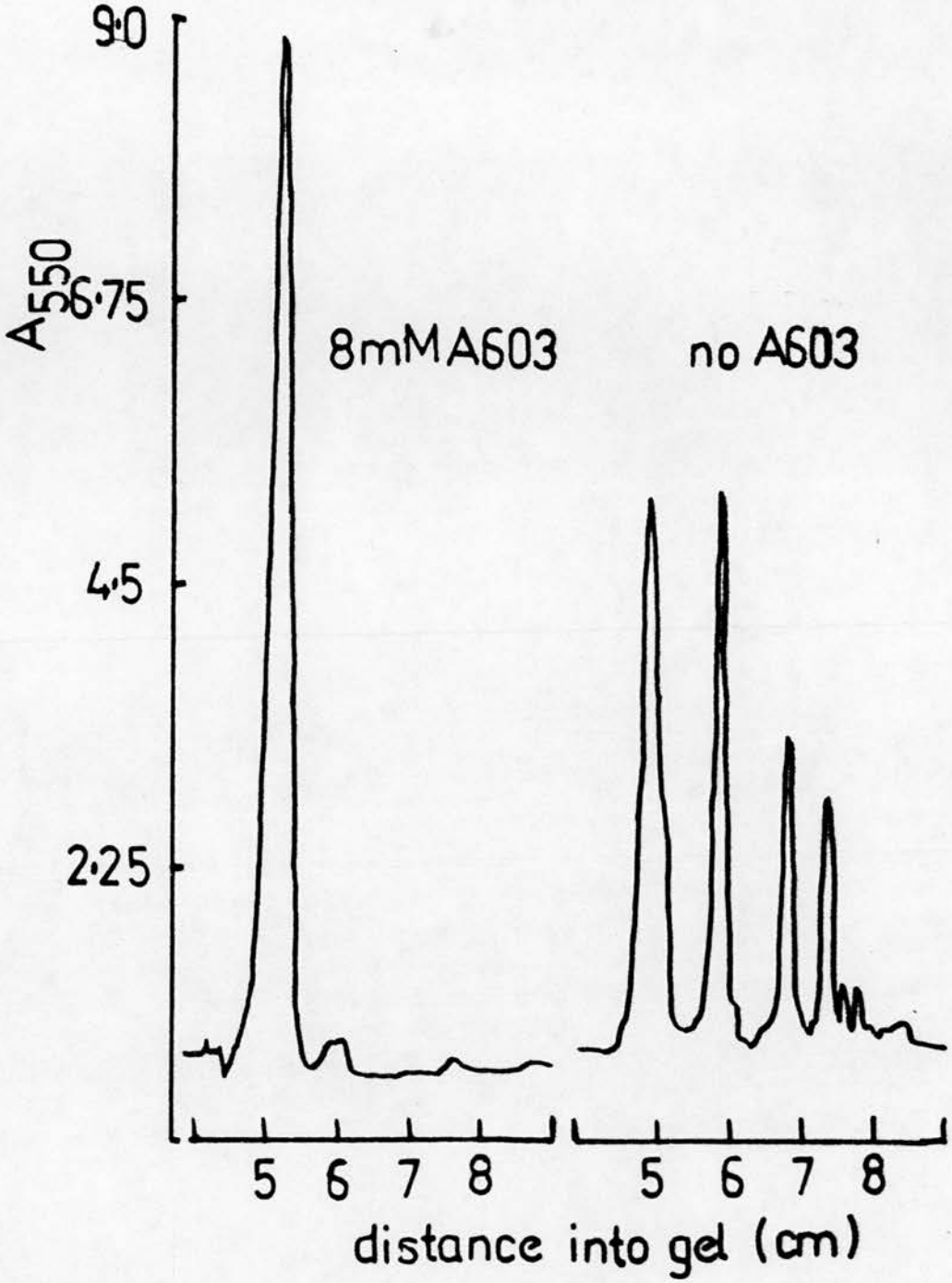


fig. 5-11 Densitometric analysis of the A_1 chain (25µg) digested with 5µg/ml trypsin in the presence or absence of 8mM A603

The implications of these results in terms of the kinetic mechanism of the toxin are discussed in Chapter 3.

Control experiments using ovalbumin as a substrate in place of the A_1 chain indicated that neither NAD^+ nor A603 affected its digestion by trypsin. In another control to ensure that neither NAD^+ nor A603 had a direct effect upon trypsin activity, digestion of the artificial trypsin substrate BAEE (α benzoyl -d,l-arginine methyl ester) was measured using a pH stat meter as described in Materials and Methods. An identical rate of digestion was obtained in the presence and absence of NAD^+ and A603.

5.2.5.2 The relationship between digestion rate and NAD^+ binding

When the rate of digestion of the A_1 chain was measured at concentrations of NAD^+ from 1mM - 20mM, the rate of digestion was found to decrease in the presence of NAD^+ (fig. 5-12). The results are presented in fig. 5-13 as a reciprocal plot of the rate of digestion as a function of NAD^+ concentration. The assumptions made for this treatment of the data are as follows.

Consider a ligand binding to an enzyme where E = A_1 chain and L = NAD^+

$$E + L \rightleftharpoons EL$$

$$K = \frac{[E][L]}{[EL]}$$

$$[E] = \frac{K[EL]}{[L]}$$

Assume that only E is digested and not EL and assume v (velocity of digestion) is given by

$$v = k[E]$$

$$V_{max} = k[E]_{max} = k[E_t] = \text{velocity measurable when } L = 0$$

$$[EL] = [E_t] - [E]$$

$$v = k[E] = \frac{kK[EL]}{[L]} = \frac{kK}{[L]} \quad ([E_t] - [E])$$

$$v = k[E_t]\,\frac{K}{[L]} - k[E]\,\frac{K}{[L]}$$

$$v = V_{max}\,\frac{K}{[L]} - v\,\frac{K}{[L]}$$

multiply by [L] and divide by v to get:

$$[L] = \frac{1}{v} \cdot V_{max}\,K - K$$

A graph of 1/v versus [L] is a straight line with intercepts -K and 1/Vmax and slope $V_{max}.K$.

this assumes that when [L] = 0, v = V_{max} (no ligand)

and when [L] = ∞ v = 0 (100% inhibition of digestion)

A K_d 4.0 ±0.4 mM was calculated from the intercept of this plot with the ordinate axis.

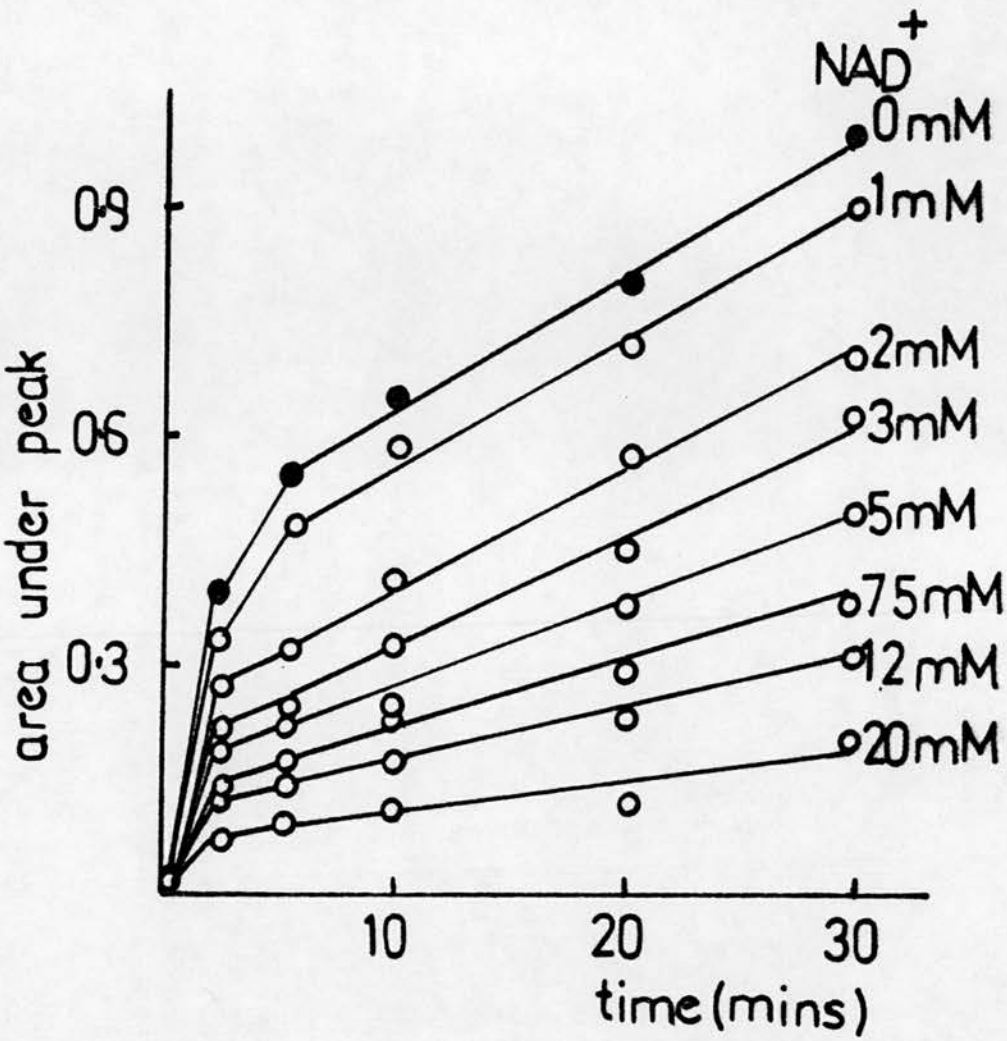


fig. 5-12 Rate of digestion of the A_1 chain in the presence of concentrations of NAD^+ from 0 - 20mM.

The rate was measured from the appearance of the digestion band of approximate molecular weight 20,000

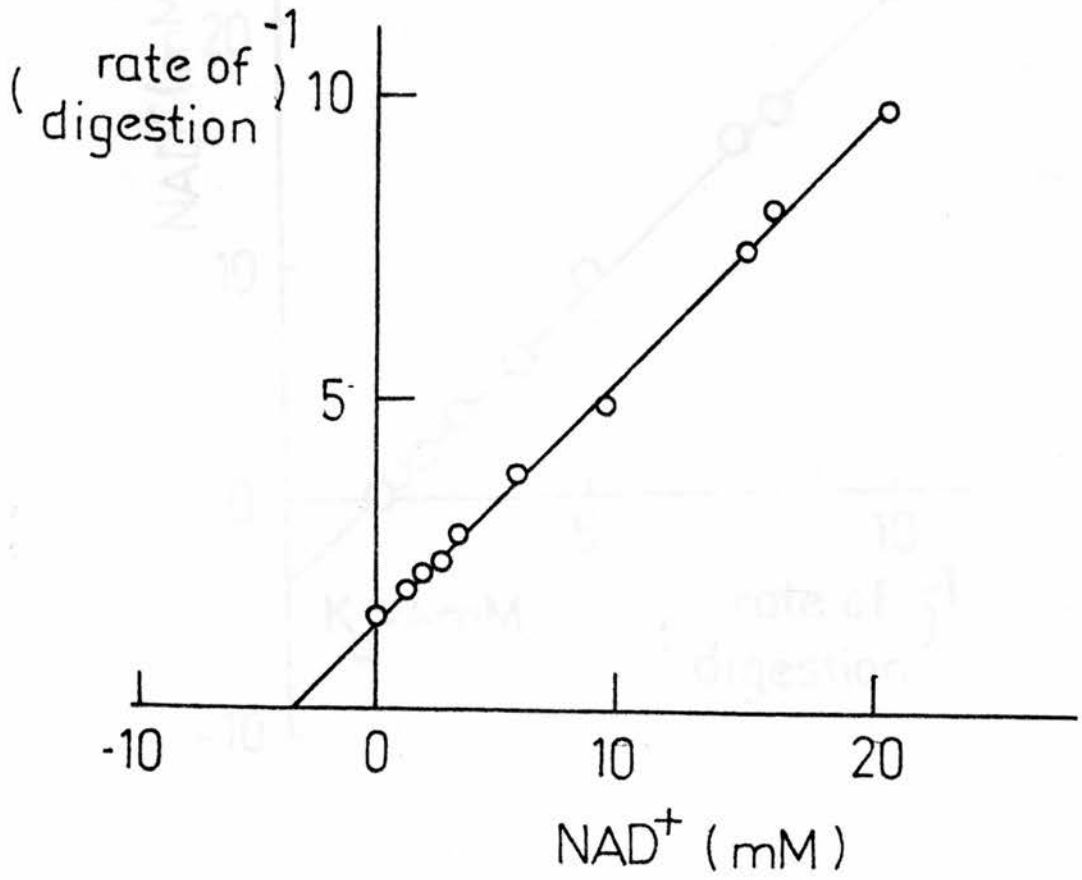


fig. 5-13 Secondary plot of NAD^+ concentration versus the rate of digestion of the A_1 chain

The rate of digestion was measured from the linear portion of the digestion rates in fig. 5-12 i.e. between 5 min. and 30 min. The rapid initial burst of digestion was not included. From this graph a K_d of 4.0±0.4mM was calculated

5.2.5.3 Binding of NAD^+ analogues

The ability of various NAD^+ analogues and derivatives to protect the A_1 chain from digestion in place of NAD^+ was studied. Samples of A_1 (25µg) in TEAN pH7.5 were allowed to stand for 10 min. at room temperature with either 10mM or 20mM additions of the following: thio NAD^+, deamino NAD^+, nicotinamide, NMN, NADP. NADH, ADP-ribose, ATP, ADP, AMP and adenine. The only molecules capable of protecting the A_1 chain from digestion by trypsin to any extent were thio NAD^+ and acetyl pyridineNAD at 10mM and above and deamino NAD^+, nicotinamide and NMN at 20mM. Protection was shown by a decrease in the extent of digestion as measured by the appearance of the largest digestion band of molecular weight approximately 20,000. NADH could also protect at 10mM. This may be due to contamination of the NADH with NAD^+ as it was not seen when the experiment was repeated using a new batch of $NADH^+$. The results are analysed in table 5.2 and compared to the results from competitive inhibition results described in Chapter Three.

compound	visible protection after 30 min.	K_i (from Chapter 3)
NAD^+	complete*	
thio-NAD^+	complete*	16mM
APAD	complete*	17mM
nicotinamide	some protection	N/D
NMN	some protection	>100mM
deamino-NAD^+	some protection	58mM
NADH	some protection	-
NADP	no protection	N/▽
ATP	no protection	-
ADP	no protection	-
AMP	no protection	-
adenine	no protection	>1000mM

Table 5-2 Protection of the A_1 chain from digestion

The ability of any molecule to protect the A_1 chain from digestion was judged from the extent of digestion of a 25µg/ml sample of the A_1 chain after 30 min. with 5µg/ml trypsin in the presence of 20mM of the sample molecule. Those marked with a star could also protect the A_1 chain from digestion at concentrations of 10mM

N/▽ = no value obtained

\- = not determined

5.3 Discussion

Here the binding of NAD^+ and the arginine substrate analogue, A603 (see fig. 3-1 for structure) to cholera toxin has been studied using a variety of methods. The overwhelming conclusion from these studies is that the millimolar concentrations of NAD^+ necessary to reach saturation have made the apparently simple task of calculating a dissociation constant for NAD^+ extremely complicated.

5.3.1 Equilibrium Dialysis

The first technique used was equilibrium dialysis. A possible problem using this technique might have been the NAD^+ Glycohydrolase activity of cholera toxin, in which NAD^+ is hydrolysed to nicotinamide and ADP-ribose. Diphtheria toxin, another ADP-ribosyl transferase, also exhibits NAD^+'ase activity, which was found by Kandel *et al.*, (1974) to be sufficiently rapid to prevent the use of equilibrium dialysis to measure binding of NAD^+ to diphtheria toxin. The dynamic dialysis technique of Colowick and Womack (1969) was used instead in this case. However, for cholera toxin, control experiments suggested that the NAD'ase activity was negligible under the conditions used. It has been suggested (Tait and van Heyningen, 1980) that some of the NAD'ase activity previously attributed to cholera toxin may in fact have been due to contamination, and that the toxin's intrinsic NAD'ase activity is of a much lower order of magnitude than its ADP-ribosyl transferase activity.

Despite a preliminary estimate of K_d = 3.0 ±2.8mM for NAD^+, the size of the error involved cast doubts on the likely accuracy of this value. After numerous efforts, the measurements of bound and free ligand continued to show errors of up to 25% between

quadruplicate samples. Possible sources of this error are outlined in section 5.2. The greatest cause of the error may be the insolubility of both the toxin and especially of the isolated A_1 chain at the concentrations of protein used in the dialysis experiments. It was virtually impossible to keep the toxin in solution in the dialysis cells except at lower concentrations (i.e. less than 1mg/ml). At these lower concentrations the percentage of NAD^+ bound would only represent 0.02% of the total NAD^+ present.

The binding of A599 or other arginine derivatives to the toxin was not studied by equilibrium dialysis as radiolabelled derivatives were not available. However it was previously reported that no binding of the ADP-ribose acceptor analogue ^{125}I-guanyl tyramine could be detected by equilibrium dialysis under conditions which would allow optimal enzyme activity for that substrate, i.e. 0.1M phosphate buffer, 60mM mercaptoethanol pH7.0 (Mekalonos *et al.*, 1979a).

5.3.2 Intrinsic fluorescence and difference spectra

Measurements of changes in the intrinsic fluorescence or absorbance of cholera toxin brought about by saturating concentrations of NAD^+ failed to produce any conclusive results. Tryptophan fluoresces at 333nm (excitation 285nm). Shifrin (1964) used the model compound indolyl ethyl-nicotinamide to show that the fluorescence of the indolyl ring is entirely quenched by its combination with nicotinamide. When diphtheria toxin and *P.aeruginosa* exotoxin A were titrated with saturating concentrations of NAD^+ (Kandel *et al.*, 1974; Chung and Collier, 1977a) there was a marked quench in their intrinsic fluorescence. The interpretation

made was that for both toxins a charge transfer complex had formed between the nicotinamide moiety of NAD^+ and a side chain of tryptophan in each protein. However, both toxins have K_d values for NAD^+ in the region of 8.9μM. They therefore require a thousand fold less NAD^+ to reach saturation. As shown in table 5-2, when a similar quenching of fluorescence was seen for cholera toxin A_1 chain, it could be explained fully by the absorbance of NAD^+ at the exciting wavelength. If a similar charge transfer complex to the one described does occur between NAD^+ and one of the two tryptophan residues in the A_1 chain (Duffy *et al.*, 1985), then it is swamped by the NAD^+ absorbance. In a similar way, the apparent difference spectra in fig. 5-3 may simply be due to inaccurate addition of NAD^+, which absorbs with a maximum at 260nm. Tyrosine and tryptophan have absorbance peaks at 281nm and 294nm respectively but these are not evident in the absorbance spectrum. Consequently, neither is any shift in their absorbance maxima, commonly seen when either residue moves into a more hydrophobic (red shift) or hydrophilic (blue shift) environment.

5.3.3 Nbf-labelled

Cholera toxin was labelled with the fluorescent probe Nbf, the amino acid derivative of which has an excitation wavelength of 438nm well outwith the absorbance of NAD^+. Under the conditions used, the Nbf probe is incorporated into lysine amino groups. Of the eight lysines in the A subunit, six are within the A_2 chain and only two in the A_1. However SDS-PAGE analysis suggested that the A_2 chain was no more heavily labelled than the A_1. The A_2 chain may be buried between the A_1 and the B_5 ring (Yamamoto *et al.*, 1985, Gill,

1976a). In such a position, its lysine residues may have been inaccessible to the Nbf probe. When the Nbf-labelled toxin or A_1 chain was treated with NAD^+, there was a change in the fluorescence spectrum of the Nbf group. This was not seen for Nbf-B subunit or for a standard solution of Nbf-myoglobin. The addition of A599 produced no changes in the Nbf-probe. The graph in fig. 5-5 implies that there is a concentration of NAD^+ at which the enhancement of fluorescence is saturated. In principle, it is possible to calculate a binding constant from these data, if the lag is ignored, as has been done in fig. 5-6 to give a K_d for NAD^+ of 40±4μM. However it is impossible to know precisely what the saturation corresponds to. When similar experiments were performed using Nbf toxin (labelled in the A subunit only) titrated with ganglioside G_{M1} (van Heyningen, 1982b) the steep slope of the titration graph obtained at low concentrations of G_{M1} was compatible with a dissociation constant of around 10^{-9}M. Here there is no such initial slope. Instead, there is a portion of the graph in which no change in the fluorescent probe is seen at all until in excess of 60μM NAD^+ had been added. The shape of the graph argues against its further use in the calculation of a K_d value. All that can be said with certainty is that the addition of NAD^+ causes a change in the environment of the Nbf probe, most likely a movement into a more hydrophobic environment.

5.3.4 Digestion of the A_1 chain with trypsin

Substrate protection from proteolysis has been reported for other ADP-ribosylating toxins (Kandel *et al.*, 1974; Tweten *et al.*, 1985) and for other unrelated proteins (Jacobs and Cunningham, 1968;

Trayser and Colowick, 1961) and has been exploited successfully in the calculation of binding constants.

Here it was found that saturating concentrations of both NAD^+ and the arginine derivative A603 could completely protect the A_1 chain from digestion with trypsin for up to 30 min. This implies that the binding of NAD^+ or of A603 to the A_1 chain results in shielding of susceptible bonds and/or leads to some major conformational change in the protein. The protection elicited by the two substrates was not additive. The K_d value of 4.0±0.4mM calculated from a secondary plot of digestion rates as a function of NAD^+ concentration agrees well with the K_m for NAD^+ for both the NAD'ase and ADP-ribosylation reactions (Moss *et al.*, 1979b; Mekalonos *et al.*, 1979). The ability of various NAD^+ analogues and derivatives to protect the A_1 chain from digestion (a tentative measure of binding affinity) also correlated with the K_i values reported in Chapter Three. The properties of the NAD^+ binding site seem consistent with the idea that it is involved in the catalytic activity of the toxin. This indirect and unusual technique has therefore allowed an estimate of the dissociation constant for NAD^+ based on the assumption that a decline in the rate of digestion by trypsin is a function of substrate binding.

The rate of digestion of the A_1 chain by trypsin was biphasic (see fig. 5-9). Similar biphasic kinetics were found when creatine kinase was digested with trypsin and the reaction followed by the release of H^+ ions (Jacobs and Cunningham, 1968) suggesting that this is not just an artefact of the SDS-PAGE method of following digestion. The linear portion of the digestion rate was measured in

each case and was found to change as a function of NAD^+ concentration.

The ability of A603 to bind to the A_1 chain in the absence of NAD^+ has implications with respect to the kinetic mechanism of the toxin. As outlined in Chapter Three, a random order sequential mechanism may exist for cholera toxin. This mechanism has also been proposed for an avian erythrocyte transferase. Diphtheria toxin and exotoxin A appear to have ordered sequential mechanisms with NAD^+ binding first.

5.3.5 The nature of the binding site

What, if anything, does this say about the nature of the binding site(s) for NAD^+ and A603? The binding of NAD^+ to the A_1 chain is of low affinity ($K_d \approx 4mM$). In fact this value is closer to the K_d of NAD^+ for dehydrogenases (typically 10^{-3} - $10^{-4}M$ (Dalziel, 1975)), in which case NAD^+ is used as a cofactor to which a hydride ion is transferred, than it is to other ADP-ribosyl transferases. For these NAD^+ is a substrate in which the bond between nicotinamide and ribose is broken. Both prokaryotic (Moss *et al.*, 1983; Gill, 1978) and eukaryotic ADP-ribosyl transferases (Osborne *et al.*, 1985; Burtscher *et al.*, 1986) have K_d values for NAD^+ in the micromolar range. There seems to be no obvious explanation for this unusually low affinity, especially when contrasted to the normal cellular concentration of NAD^+ of around 70-100μM (Gill, 1975).

The binding of NAD^+ to the toxin seems to involve recognition of the whole NAD^+ molecule, with nicotinamide especially important. Similar results were reported for diphtheria toxin and exotoxin A and for dehydrogenases which interact with the whole NAD^+ molecule.

There have been unsubstantiated reports from secondary structure predictions that cholera toxin, diphtheria toxin and *E.coli* LT may all have a βαβα sequence of secondary structure (Duffy and Lai, 1986; Yamamoto *et al.*, 1984). This structure is proposed to resemble the so-called "Rossman fold", an alternating α helix and β-sheet structure which constitutes the NAD^+ binding region of lactate dehydrogenase (Rossman *et al.*, 1974) and other dehydrogenases. However, direct evidence of the secondary structure of exotoxin A derived from X-ray crystallography (Allured *et al.*, 1986) failed to detect any such structure. The most notable structure feature of the active site domain was an extended cleft. It seems unlikely then that diphtheria toxin contains a Rossman fold-type structure. Exotoxin A and diphtheria toxin fragment A have virtually identical specific activities, K_m values for NAD^+ and Ef-2, sensitivities to inhibition and sequential mechanisms (Chung and Collier, 1977b). These results all suggest a high degree of homology in the structures of the active sites on the two peptides. It remains unproven then, that cholera toxin has a βαβα structure as part of its binding site.

The NAD'ase activity of cholera toxin implies that when NAD^+ is bound to the A_1 chain, its nicotinamide-ribose linkage is accessible to nucleophilic substitution by water. It seems probable, therefore, that it might also be highly reactive with an appropriately placed nucleophilic group on N_ϵ (or an analogue thereof). A working model proposed by Tait and Nassau (1984) agrees with this. On the basis of results using a number of artificial arginine derivatives as substrates for ADP-ribosylation, the active

site of the toxin was inferred to comprise a hydrophobic pocket responsible for efficiently binding the substrate, with a hydrophilic region associated with the site at which the ADP-ribosylation reaction occurs. This model does not explain why the binding of NAD^+ should be of such low affinity. A segment of the A_1 chain, notable for its high concentration of hydrophobic residues has been proposed to be a transmembrane segment involved in the insertion of the A_1 chain into the plasma membrane (Yamamoto *et al.*, 1984). As previously noted (Chapter Three), covalently cross-linked toxin can still activate adenylate cyclase and may only require to penetrate the membrane to catalyse its reaction. Taken together with the hydrophobic nature of the ADP-ribose acceptor (Tait and Nassau, 1984; Mekalonos *et al.*, 1979a; Gilman, 1984) the suggestion is that the reaction occurs close to the plasma membrane. Perhaps this has some unexplained bearing on the affinity for NAD^+ *in vivo*.

Chapter Six Photo affinity labelling of cholera toxin with NAD^+

6.1 Introduction

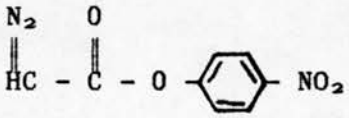
The studies in the preceding chapters have provided some ideas about the interaction of NAD^+ with cholera toxin, namely the possible kinetic mechanism and the affinity of binding. But they have given no idea as to the location of the NAD^+ binding site within the toxin's A_1 chain. Since cleavage of the disulphide bond between the A_1 and A_2 chains is required for activity, and since S-alkylated A_1 retains its catalytic activity, the portion of the molecule near the only cysteine residue in A_1 at position 191 (but not the SH group itself) has been suggested as a candidate for the A_1 chain's active site (Wodnor-Filipowicz and Lai, 1976; Xia *et al.*, 1984). Certainly there is a change in the conformation of the A_1 chain following reduction (de Wolf *et al.*, 1985). However, comparison of the amino acid sequence of cholera toxin's A_1 chain with that of the closely related *E.coli* LT A_1 chain indicated that the amino termimal amino acids showed the greatest homology with long stretches of identical sequence and that there was greater divergence towards the carboxyl terminus (Duffy *et al.*, 1985; Yamamoto *et al.*, 1984; Spicer and Noble, 1982; Dykes *et al.*, 1985).

The classical approach to acquiring information about the residues at the active site of an enzyme has been the use of general and specific reagents for chemical modification of active site residues, commonly referred to as affinity labelling (Singer, 1967; Shaw, 1970; Wold, 1977). Ideal labelling reagents should react not only with nucleophiles but also with the less reactive but ubiquitous hydrophobic regions, such as C-H bonds, of proteins.

This high activity should be masked but capable of activation once the label is in place at the active site.

Photogenerated affinity labels satisfy both these requirements. This form of labelling usually requires the modification of the natural ligand by the incorporation of a chemically inert but photolabile group. Most of these labels are electrophilic species as they have to compete between a nucleophile at the binding site and the water in which the labelling reaction occurs. Irradiation can give rise to two general classes of species, each of which is produced by the homolytic cleavage of chemical bonds. The absorption of ultraviolet or visible radiation may lead to fragmentation either at a single bond, resulting in the formation of two free radicals or a diradical, or at a double bond or nitrogen, which results in a carbene or nitrene (Bayley and Knowles, 1977). These highly reactive species can then go on to react in a number of ways with adjacent residues, including addition to double bonds, insertion into single bonds, hydrogen abstraction, etc. (for a more detailed account see Bayley and Knowles, 1977 and Chowdry and Westheimer, 1979).

The first photo affinity label to be used was p-nitro phenyl diazo acetate, used in the identification of



p-nitro phenyl diazo acetate

chymotrypsin's active site serine residue. Other diazo acyl derivatives have been synthesised for trypsin (Hexter and Westheimer, 1971) and subtilisin (Stefanovsky and Westheimer, 1973).

p-Azidophenyl-acylbromide was used to study glyceraldehyde-3-phosphate-dehydrogenase (Hixson and Hixson, 1975), while 8-azido cyclic-AMP was successfully used in the study of cyclic-AMP binding proteins (Hayley, 1975). A number of NAD^+ analogues have been synthesised including arylazido-β-alanine NAD^+ and 8-azido adenine NAD^+ which were successful substitutes for NAD^+ (Chen and Guillory, 1977; Koberstein, 1976). However, previous attempts at synthesising suitable photolabile NAD^+ derivatives were not so successful. Neither 3-diazo acetoxymethyl NAD^+ (Browne *et al.*, 1971) nor 3-azido NAD^+ (Hixson and Hixson, 1973) proved to be suitable when used to label yeast alcohol dehydrogenase. They both had reduced affinities for the enzyme, which binds NAD^+ relatively loosely anyway ($K_d = 5 \times 10^{-4}M$). There was only a low level of incorporation of the label, much of which may have been non-specific. These examples illustrate the problems inherent in photolabelling of "loose" rceptor binding complexes. In principle, if the dissociation constant is millimolar or below, and binding of the analogue is competitive with the natural ligand, specific labelling will be seen provided the lifetime of the reactive species generated is short (10^{-5} sec or less).

The problems of reduced affinities of some photolabile substrate analogues can be avoided if the natural ligand itself is used as the photolabel. In this way there is no uncertainty about whether the label behaves in an identical way to the natural ligand. If the natural ligand has a functional group or aromatic moiety then it may make an excellent photolabel. Unsaturated keto steroids have been used to identify hormone receptor proteins including glucocorticoid

(Govindan and Gronomeyer, 1984) and progesterone receptors (Birnbaumer *et al.*, 1983) and also androgen-binding protein (Taylor *et al.*, 1980). Underivatised thyroid hormones make good labels of thyroid hormone binding proteins (van der Walt *et al.*, 1982). Nucleotides and cyclic nucleotides make very efficient labels (Antonoff *et al.*, 1976). When ATP was used to photolabel the apparent catalytic site on the heavy chain of myosin, up to 0.5 moles of ATP were incorporated per mole of protein (Manuta and Korn, 1981).

It was recently shown that irradiation of mixtures of diphtheria toxin fragment A and [carbonyl-^{14}C] NAD$^+$ with ultraviolet light induced efficient transfer of the radiolabel to position 148, corresponding to glutamate in the unmodified protein (Carroll *et al.*, 1980; Carroll and Collier, 1984). The photoproduct (an α-amino-ϑ-(6-nicotinamidyl) butyric acid residue) contained the entire nicotinamide moiety of NAD$^+$ linked via its number 6 carbon to the decarboxylated ϑ-methylene carbon of glutamate 148 (Carroll *et al.*, 1985). No portion of the ADP-ribose moiety had been incorporated. When site directed mutants of diphtheria toxin fragment A were manufactured, in which glutamate 148 was replaced by aspartate, the ADP-ribosylation activity of fragment A was abolished (Tweten *et al.*, 1985). These results indicate that glutamate 148 must be at or near the catalytic centre of the toxin.

Here the photoaffinity labelling of cholera toxin's A_1 chain with underivatised NAD$^+$ has been attempted using a modification of Collier's method. The bulk of this work was performed in the Department of Microbial Biochemistry, Glaxo Group Research, Ltd.,

Greenford, Middlesex. Some of these experiments used large amounts of cholera toxin and radiolabelled NAD^+, both of which are very expensive. Because of this, limitations on the scale of some of the studies were imposed by financial constraints.

6.2 Results

6.2.1 Photo-induced incorporation of NAD^+ into cholera toxin

Photoaffinity labelling experiments were performed as follows. Mixtures of cholera toxin, 60µM in TEAN buffer, pH7.5 and NAD^+, 1mM - 16mM containing 25µCi (25nMoles) [$4^{-3}H$] NAD^+ in the presence of 20mM DTT, were allowed to stand for 10 min. at 4°C. Droplets of 100 - 150µl were then placed on a parafilm-coated micro-titre plate floating in an ice bath. Experiments were performed at 4°C to minimise breakdown of the NAD^+ by the toxin's NAD'ase activity. The droplets were irradiated at a distance of 5cm using a CAMAG 100w dual wavelength ultra violet lamp at 254nm. At the times indicated, 20µl aliquots were removed, and firstly treated by precipitating any covalent protein-ligand formed on Whatman 3mm paper impregnated with 12% trichloroacetic acid (TCA), 0.1M phosphoric acid. However there seemed to be some non-specific adsorption of NAD^+ onto the filter paper resulting in high background levels. These high backgrounds were reduced using the following procedure. Aliquots were removed as described, then diluted with 25µl 6M urea containing 2-5% BSA. The urea was found necessary to dissociate any non-covalently complexed protein and ligand. Samples were then precipitated with 12% TCA/phosphoric acid, centrifuged and the pellets washed a further three times.

In preliminary experiments to determine the time-course of incorporation, [$4^{-3}H$] NAD^+ was replaced in some experiments with [adenine-$2,8^{-3}H$] NAD^+. Results, presented in fig. 6-1, show that incorporation of both adenine- or nicotinamide-labelled NAD^+ continued over 2 hours. When the experiment was repeated for up to

4 hours, the rate of incorporation was seen to level off (fig. 6-2). Varying the pH from 6.5 - 8.5 had no significant effect on the overall level of incorporation. The effect of varying the NAD^+ concentration is shown in fig. 6-3. When the NAD^+ concentration was increased up to 16mM there was a small increase in both the rate and extent of incorporation. A greater increase may have been prevented by a concomittant increase in optical shielding caused by the absorbance of NAD^+ at 260nm. As evident in figs. 6-1 - 6-3, the level of incorporation of NAD^+ was around 0.05 - 0.20 moles per mole of toxin.

The concentrations of reagent are important in the design of photolabelling experiments. If absolute specificity is required then a high protein concentration and a low ligand concentration should provide this, at the expense of a higher extent of labelling. The converse situation, with excess ligand (at a concentration greater than the dissociation constant) over protein should increase the extent of labelling. However, non-specific labelling (whereby labelling occurs outside the binding site, also called 'pseudo affinity labelling' (Ruoho *et al.*, 1973)) can occur when high concentrations of ligand are used. Non-specific labelling may especially be a problem for loosely bound ligands, as is the case here for NAD^+ and cholera toxin. The concentration of NAD^+ chosen was therefore 5mM, (K_d = 4mM). The photolabelling was also performed in the presence of scavengers, which are intended to destroy all photogenerated intermediates at places other than the

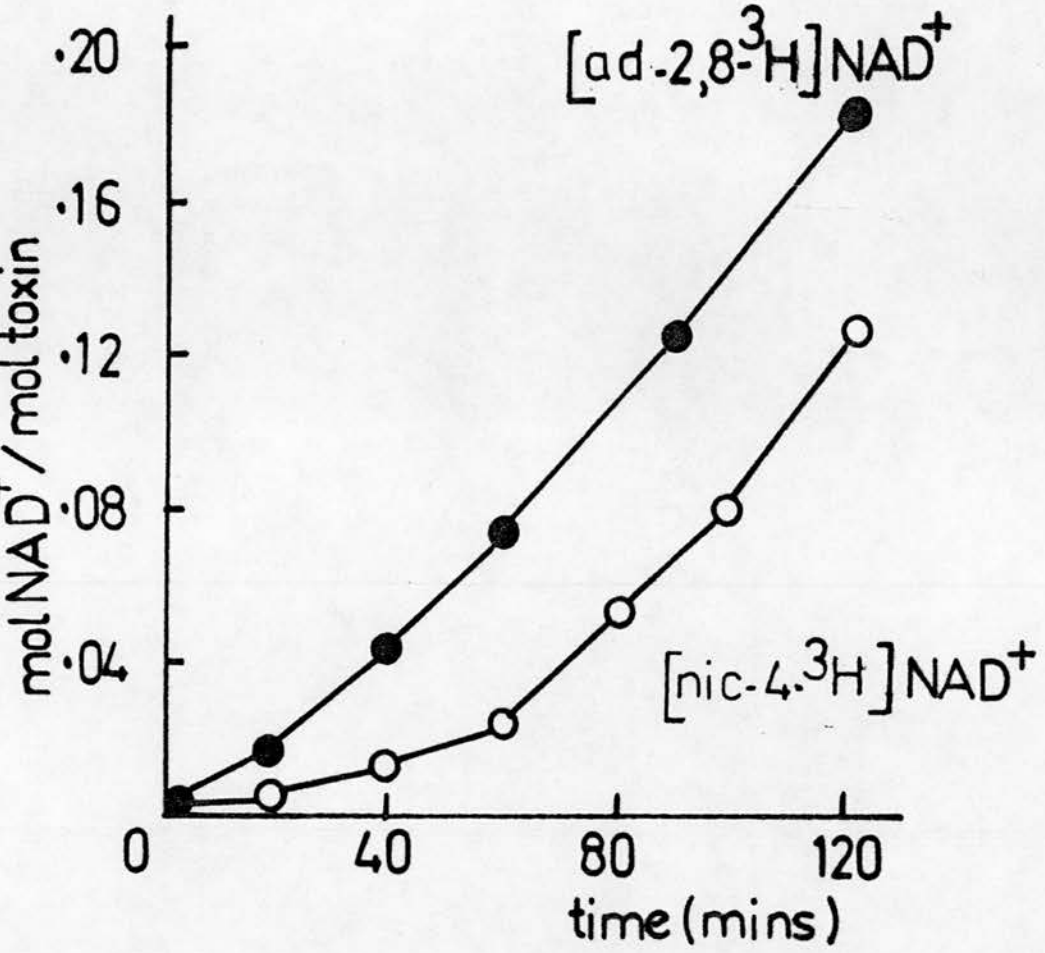


fig. 6-1 **Photoinduced incorporation of NAD^+ as a function of time**

Samples contained cholera toxin, 60µM in TEAN buffer, pH7.5, 20mM DTT and 5mM NAD^+ containing either 10µCi (36nM) [Ad-2,8-^{3}H] NAD^+ or 25µCi (25nM) [nic-4-^{3}H] NAD^+. Samples were irradiated and treated as described in the text.

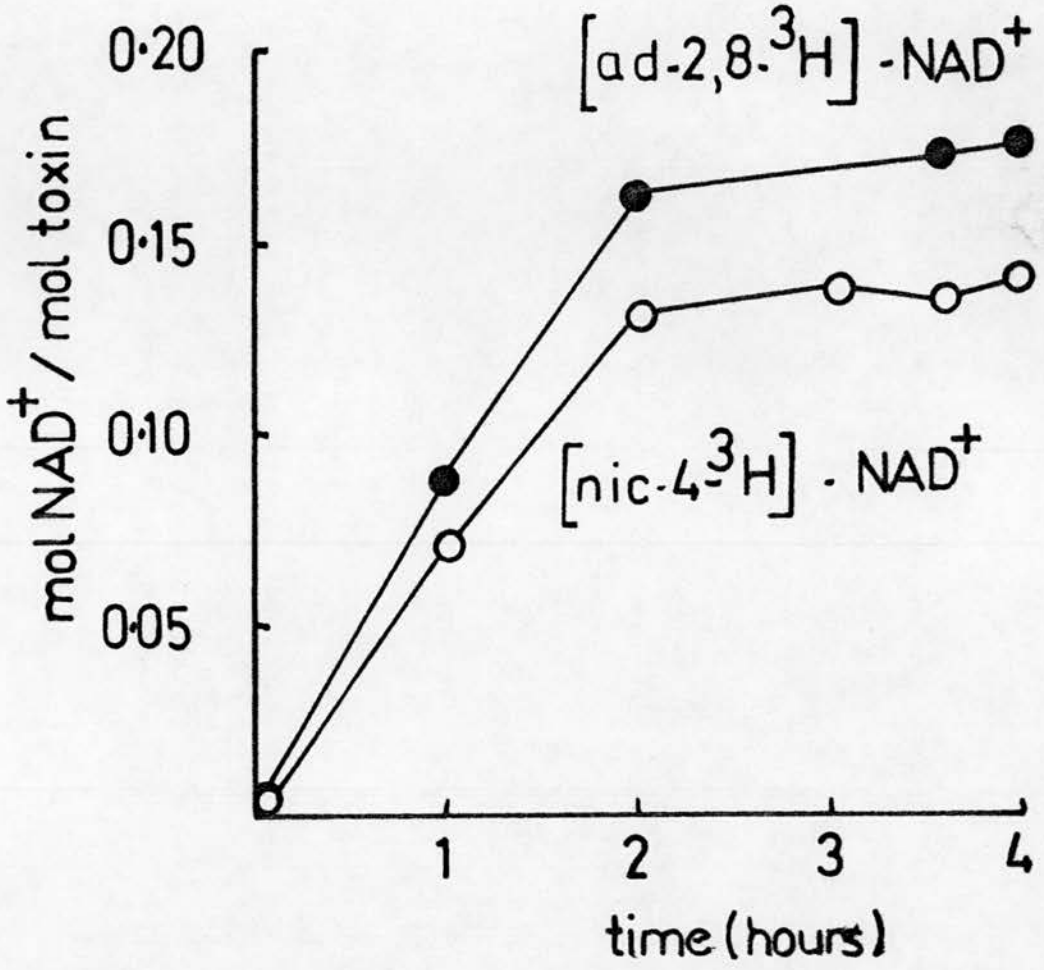


fig. 6-2 Photoinduced incorporation of NAD^+ as a function of time

Samples contained cholera toxin, 60µM in TEAN buffer, pH7.5, 20mM DTT and 5mM NAD^+ containing either 10.8nM (30µCi) [Ad-2,8-^{3}H] NAD^+ or 30nM (30µCi) [nic-4-^{3}H] NAD^+. The samples were treated as described in the text.

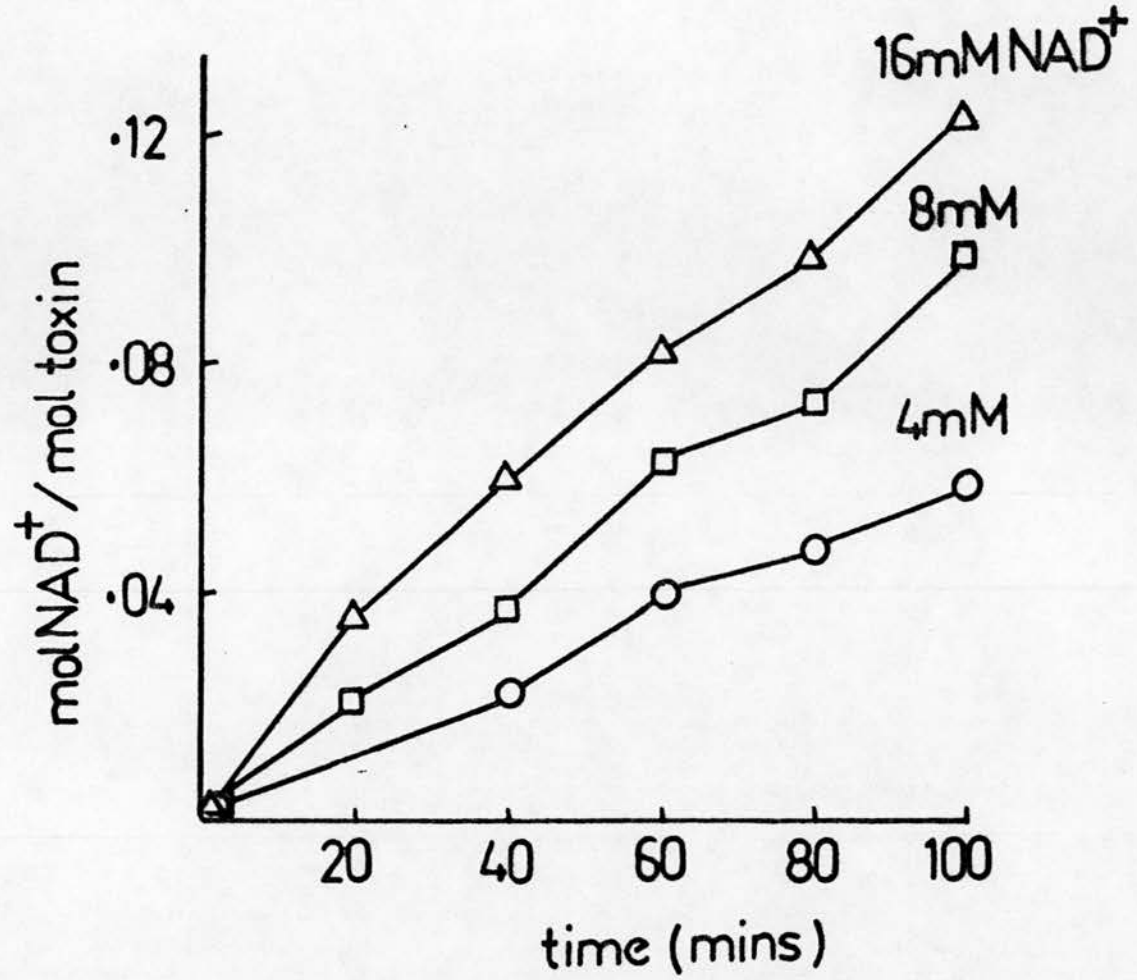


fig. 6-3 The effect of NAD^+ concentration on photoinduced incorporation

Samples contained cholera toxin, 60μM in TEAN buffer, pH7.5, 20mM DTT and 4-16mM NAD^+ containing 3.8% [nic-4-^{3}H] NAD^+. Samples were treated as described.

binding site i.e. free in solution. The 'scavengers' used here were DTT and Tris (Rudnick *et al.*, 1975; Maasen and Möller, 1974). The concentrations of Tris and DTT used did not absorb significantly at the exciting wavelength. In general, non-specific binding is not saturable (Browne *et al.*, 1971; Katzenellenbogen *et al.*, 1974; Cooperman *et al.*, 1975). The time course in fig. 5-2 therefore implies that the incorporation here is a specific, if very slow event.

When incorporation of radiolabelled NAD^+ was studied in the presence of 100mM unlabelled NAD^+ it was found that the level of incorporation of radioactivity was reduced to the level of the background controls (namely, mixtures of cholera toxin and NAD^+ incubated under identical conditions as the samples but not exposed to ultraviolet light, and irradiated controls containing everything except cholera toxin). A similar inhibition of radioactive incorporation was seen when irradiation was performed in the presence of 100mM nicotinamide or 100mM APAD (acetyl pyridine adenine dinucleotide, a competitive inhibitor of NAD^+). This indicates that the radiolabelled NAD^+ binds to the same site as NAD^+, presumably the active site of the toxin.

6.2.2 Stability of protein and ligand

Prolonged photolysis can result in such undesirable effects as slow non-specific labelling by photolysis products and destruction or alteration of the binding site. Exposure of NAD^+ to short-wave ultraviolet light will eventually lead to its degradation to leave nicotinamide and ADP-ribose (Carter, 1950; Seraydarian *et al.*, 1954). Exposure of proteins to ultraviolet light of below 300nm may

lead to damage, denaturation and/or protein-protein cross-linking and aggregation (Jori and Spikes, 1978). In view of the long exposure times of up to 2 hours, controls were performed to check the stability of the protein and ligand over this time. Samples of cholera toxin were irradiated in TEAN buffer, pH7.5, 20mM DTT for up to 2 hours. The activity of the irradiated samples was then compared with samples treated in an identical way but not exposed to radiation. The irradiated samples retained 80 - 100% of their activity. The integrity of the NAD^+ was checked as follows. Mixtures of cholera toxin and NAD^+ were allowed to stand on ice, without irradiation, for 2 hours. Samples containing NAD^+ but no toxin were irradiated for up to 2 hours. Aliquots from both were then analysed by tlc as described in section 2.9. Breakdown of NAD^+ into nicotinamide under these conditions was below 10%.

The lamp used for irradiation was of relatively low intensity, 8w, when compared to the 1kw mercury-xenon lamps commonly used for photo-affinity labelling experiments using steroids (Gronomyere, 1985). This may account for the long time courses of incorporation (incorporation of NAD^+ into diphtheria toxin was virtually complete after 30 min. using a 15w lamp (Carroll and Collier, 1984) while photoincorporation of the herbicide analogue N-4-azidobenzoyl-3,4-dichloro aniline is half complete in 20 sec (Bayley and Knowles, 1977). Rapid photolysis may be more important in the case of chemically unstable ligands and derivatives, which may undergo slow irradiation-independent degradation. Compared to the photo affinity label arylazido-β-alanine NAD^+ for instance (Chen and Guillory, 1977), NAD^+ is relatively stable. The stability of both protein and

ligand may even be increased on binding owing to screening effets caused by the light absorption of both. Alternatively the sensitivity of the ligand to irradiation may increase on binding to protein.

6.2.3 Specificity of NAD^+ incorporation

To determine whether or not photo-induced incorporation of NAD^+ was evident for any other proteins, irradiation of several proteins with NAD^+ was performed. These proteins included lactate dehydrogenase (which binds NAD^+), BSA, alkaline phosphatase and trypsin. All of the proteins were irradiated at concentrations of 5mg/ml in TEAN buffer, pH7.5 with 20mM DTT, and 5mM NAD containing [4-^{3}H] NAD^+. No incorporation of radioactivity was seen for any of these proteins. In addition, a sample of the *E.coli* LT prepared in Chapter Four was similarly treated. There was no incorporation of radioactivity either with nicked or un-nicked toxin. This result may not, however, be significant as the preparation of LT consistently failed to show activity in a number of systems (see Chapter Four).

6.2.4 Activity of photolabelled toxin

A standard method for determining whether labelling has occurred is to check the activity of the enzyme after removal of unbound ligand. If the enzyme's active site is blocked, it should no longer be active (Katzenellenbogen *et al.*, 1974). Samples of irradiated toxin (1mg total) were therefore dialysed against TEAN buffer, pH7.5, to remove excess unbound ligand and then applied to a 2.0ml column of NAD^+ agarose (Sigma) equilibrated in the same buffer. In theory, labelled toxin should pass straight through the column

unretarded. Unlabelled toxin should bind to the NAD^+ agarose, from which it can be eluted with 10mM NAD^+. In practise, all of the toxin applied to the column passed through unretarded. It appeared at first that dialysis may have failed to remove all of the non-covalently bound NAD^+. The dialysis was repeated, this time in the presence of 2.5M urea. Again the toxin was applied to the column but again it all passed through unretarded. It seems unlikely that all of toxin had been covalently labelled with NAD^+ (unlabelled NAD^+ was used which could not therefore be readily quantified following TCA precipitation). In fact, when tested using the ADP-ribosylation assay described in section 2.9, the toxin showed close to full activity. Samples of non-irradiated toxin failed to bind to the NAD^+ agarose column either suggesting the problem to be caused by the affinity agarose itself. Even if the NAD^+ agarose column had functioned as intended, it is unlikely that the amount of photo labelled toxin formed under these conditions would have been sufficient to allow an accurate estimate of its activity. The level of incorporation of NAD^+ was typically 0.05-0.1mol NAD^+/mol toxin over 1 hour (from fig. 6-1). The ADP-ribosylation assay used for determining activity requires around 100µg in total for an accurate estimate of activity. Upwards of 5mg cholera toxin would have to be irradiated to provide sufficient photolabelled toxin for an accurate activity estimate. Financial constraints imposed by the expense of both toxin and radiolabel did not permit this experiment to be repeated using these concentrations of protein.

It is not always the case that a photoaffinity-labelled enzyme will show a complete loss of activity (although it should be if an

active site residue is involved). Because of the lack of chemical specificity of many photoaffinity labelling reactions, the labelled material may be composed of a mixture of closely related products, some of which may retain partial biological activity. Likewise, the presence of ligand may increase the sensitivity of some proteins to photolytic inactivation (Cysyk and Prusoff, 1972) and may mediate photooxidation or other photosensitized destructions (Jori, 1975; Laustrait and Hasselman, 1975). In those cases the demonstration of a loss of biological activity would not necessarily imply that photoaffinity labelling of the active site had occurred.

6.2.5 Location of the photoincorporated NAD^+ within the toxin

It is the A_1 chain of the toxin which binds NAD^+ and which contains the catalytic activity of the toxin. It was important to show that it was in fact the A_1 chain into which NAD^+ had been incorporated.

Samples of 50µM cholera toxin in TEAN, pH7.5, 20mM DTT and 5mM NAD^+ with either 16.7nM [4-^{3}H] NAD^+ or 16.7nM [A-2,8-^{3}H] NAD^+* in a total volume of 100µl were irradiated for 2 hours. The samples were subjected to 12% SDS-PAGE, stained, destained and soaked in New England Nuclear "en^{3}Hance" fluorographic enhancer before autoradiography. After 14 days the autoradiograph was developed and is shown in fig. 6-4. All of the radioactivity had migrated with the A_1 chain of apparent molecular weight 22,000. In addition to confirming that the NAD^+ was located in the A_1 chain, this result confirms the covalent nature of the labelling, i.e. the label is retained despite electrophoresis in the presence of SDS and extensive washing of the resulting gel with denaturing solvents.

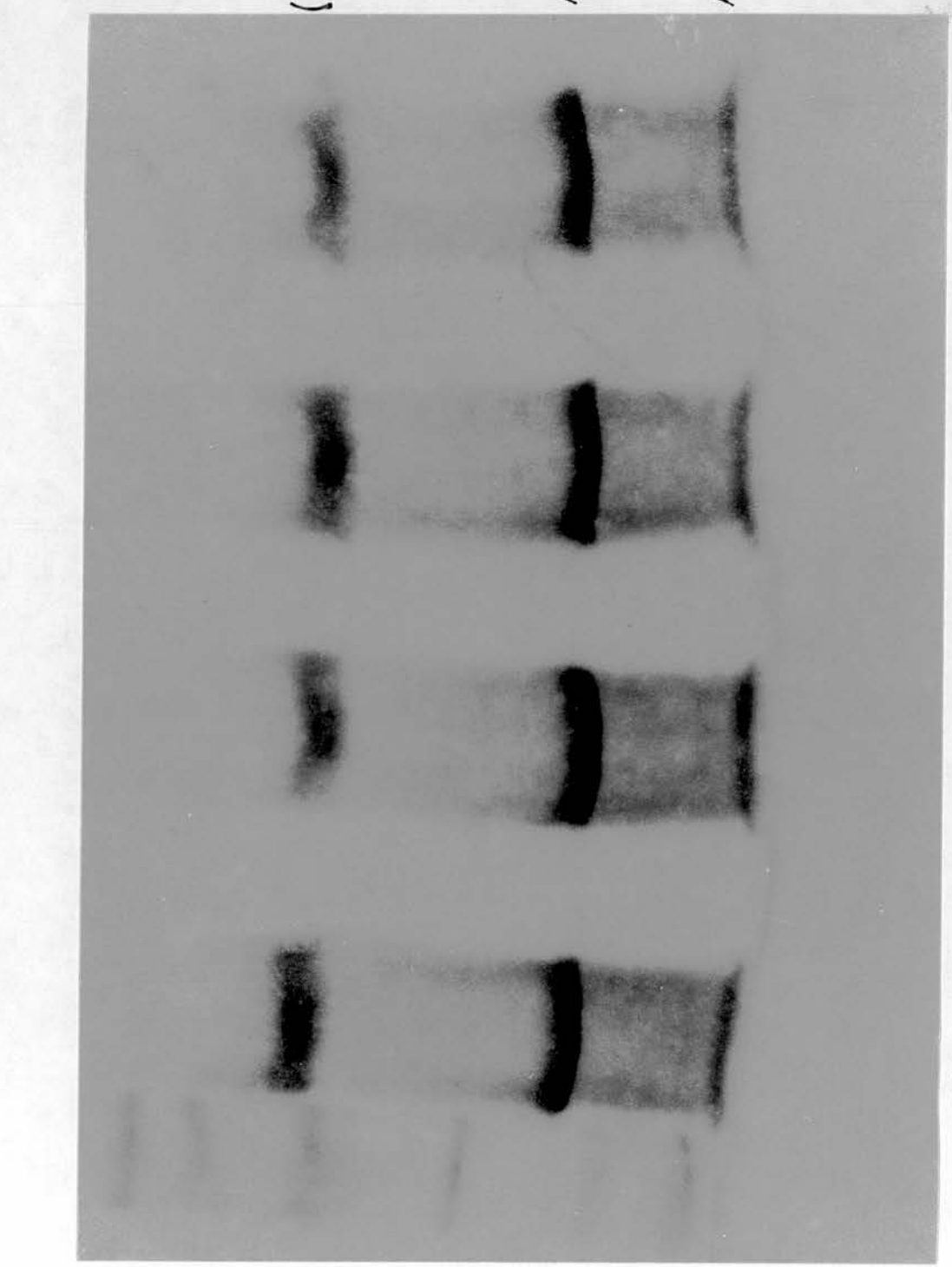
* 16.7nM [4-^{3}H]NAD^+ = 16.7µCi
16.7nM [A-2,8-^{3}H]NAD^+ = 56µCi

Although the NAD^+ was incorporated into TCA-precipitable material in section 6.2.1 it could have been argued that some of the ligand had merely become physically entrapped in the protein precipitate.

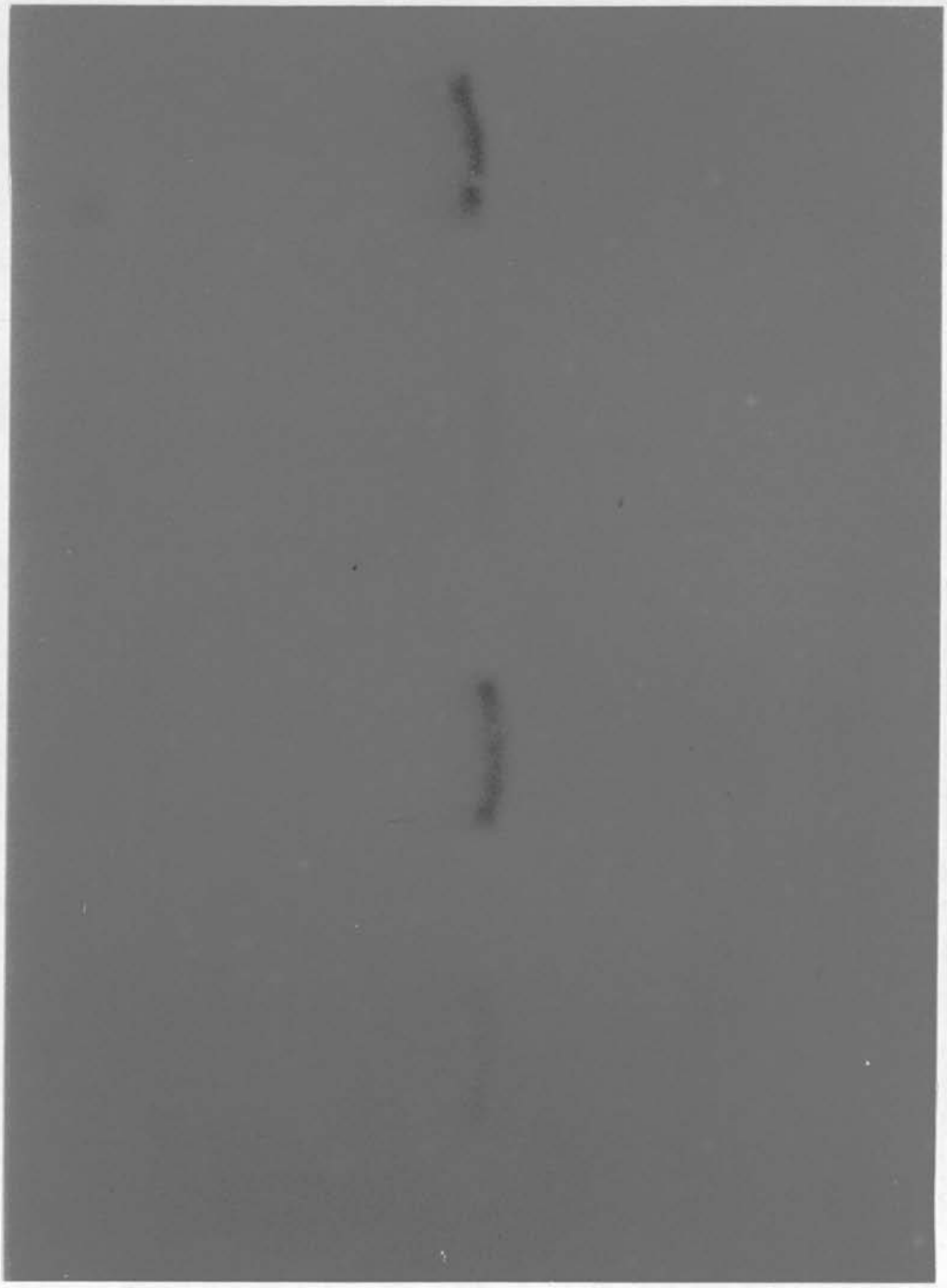
fig. 6-4 12% SDS-PAGE of radiolabelled toxin showing the incorporation of radioactivity into the A_1 chain.

The autoradiograph in fig(b) was developed for 14 days. | Lanes (1) and (3) show cholera toxin irradiated in the presence of 16.7µCi [nic-4-^{3}H] NAD^+, (2) and (4) were irradiated with 56µCi [Ad-2,8-^{3}H] NAD^+ on a coomassie blue stained gel in fig(a)

(a)



(a)



6.2.6 Isolation of a radiolabelled peptide from the A_1 chain

A sample of irradiated toxin was chromatographed on a column of Sephadex G75 in 5% formic acid. This treatment has previously been shown to separate the toxin into its constituent subunits (Lai *et al.*, 1976). The five B subunits, which remained as an aggregate under these conditions (they can be separated by boiling in dilute SDS) were eluted first, closely followed by the A_1 chain (as the irradiation was performed in the presence of DTT, the disulphide bridge linking the A_1 and A_2 chains will have been reduced). As shown in fig. 6-5 all of the radioactivity migrated with the A_1 chain. This again points to the covalent nature of the NAD-protein linkage. Fractions containing radioactivity were pooled and freeze-dried.

The freeze-dried A_1 chain was then taken up in 3ml TEAN buffer, pH7.5 and digested with trypsin (2%w/w) for 3 hours at room temperature. The digested peptide was then applied directly to a column of Sephadex G25 (2.2 x 90cm) equilibrated with 50mM ammonium formate, pH3.0. One main radiolabelled peak was recovered as shown in fig. 6-6.

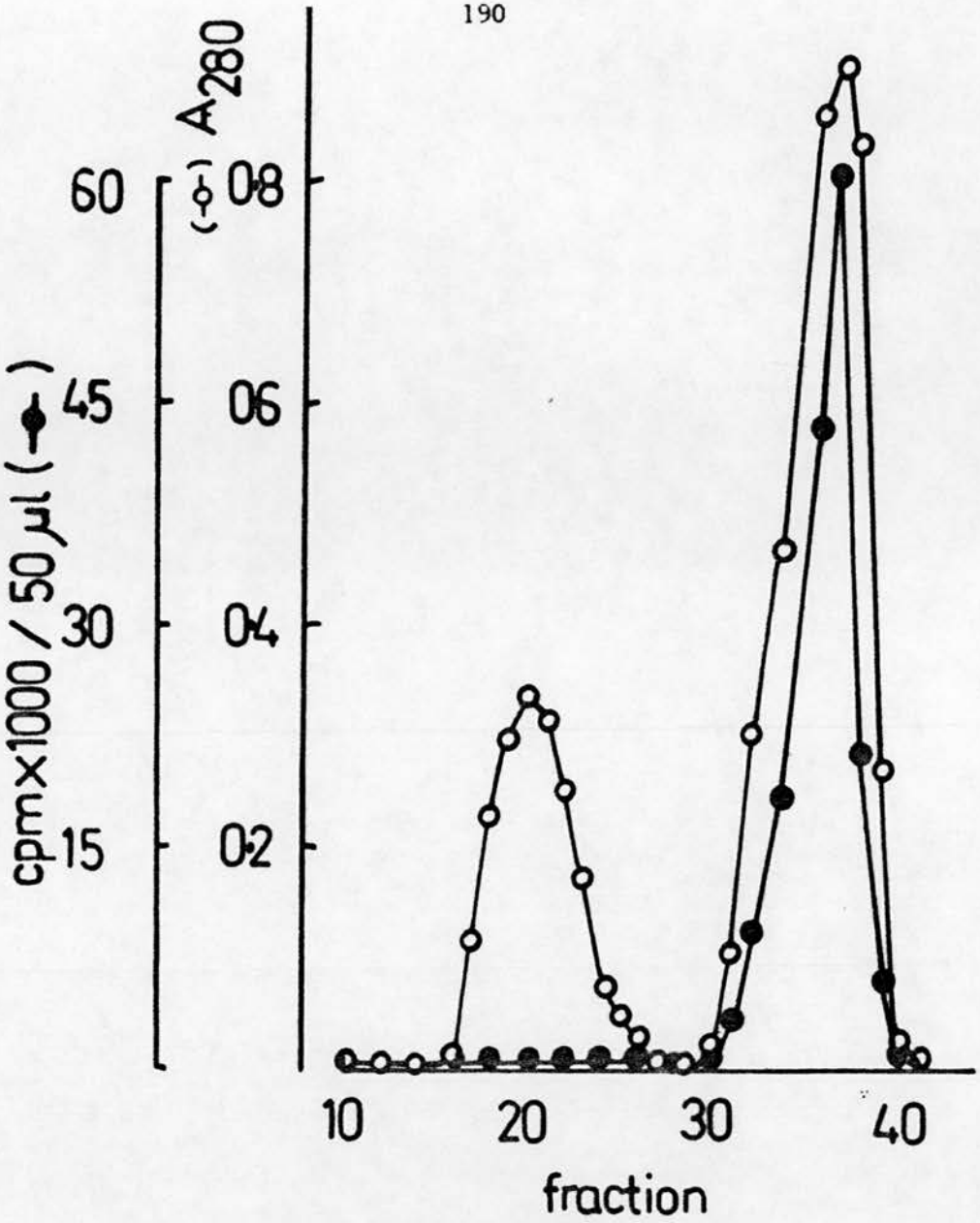


fig. 6-5 Isolation of the radiolabelled A_1 chain

A total of 5mg cholera toxin was mixed with 5mM NAD containing 175nM [4-^{3}H] NAD$^+$* in a total volume of 750µl TEAN buffer, pH7.5 containing 20mM DTT. The mixture was irradiated as described then dialysed extensively against TEAN buffer pH7.5 until no more radioactivity was recovered in the dialysate. Formic acid was added to a final concentration of 5% and the sample was loaded onto a column of Sephadex G75 (1cm x 110cm) pre-equilibrated in 5% formic acid. The column was run at 20ml/h and fractions of 2.0ml were collected and assayed for radioactivity and for absorbance at 280nm

* 175nM [4-^{3}H] NAD$^+$ = 175µCi

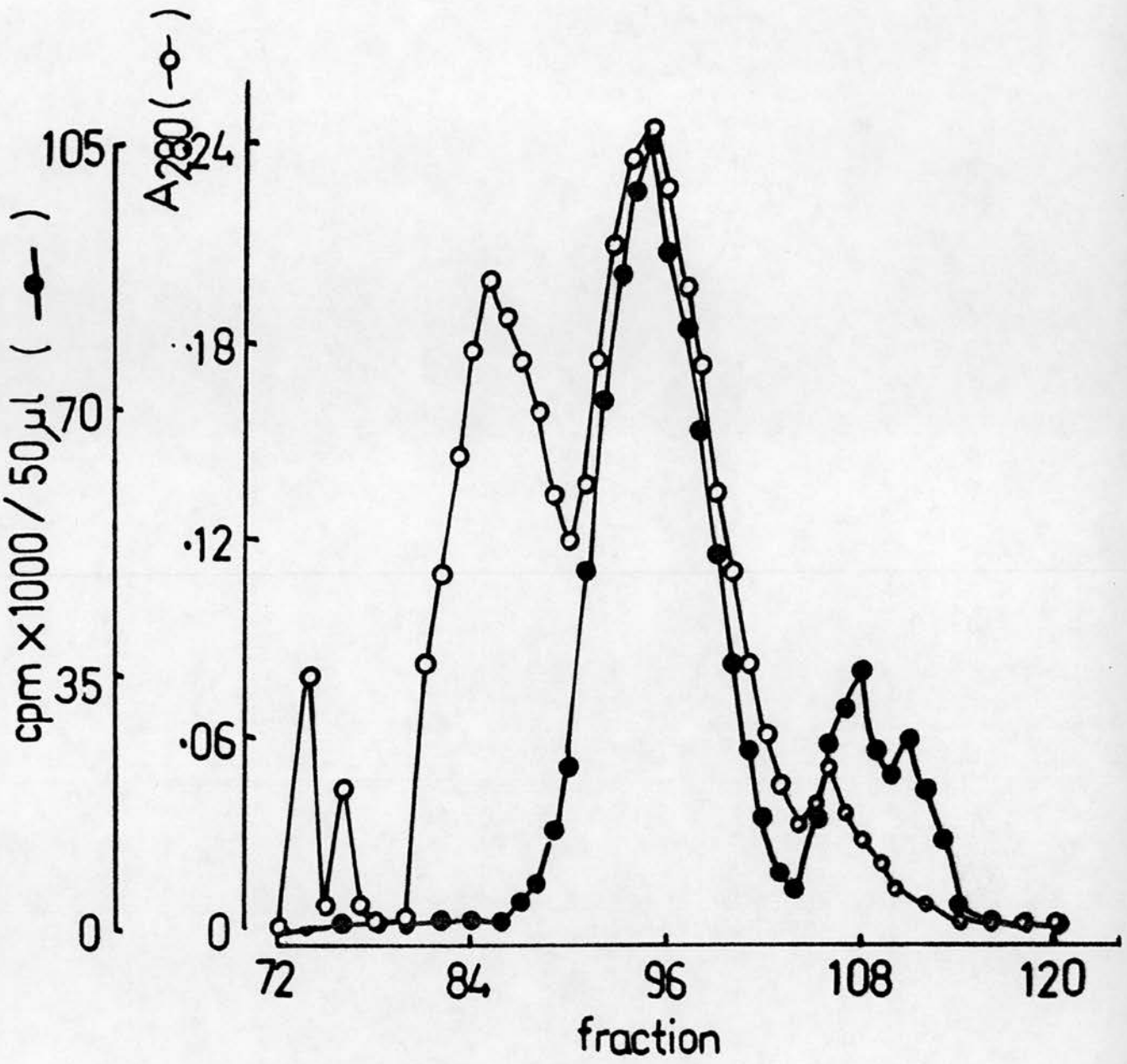


fig. 6-6 Column chromatography of tryptic peptides from radiolabelled A_1 chain

Digested radiolabelled A_1 chain was applied to a column of Sephadex G25 (2.2 x 90cm) and run at 10ml/h. Fractions of 2ml were collected and assayed for radioactivity and for absorbance at 280nm

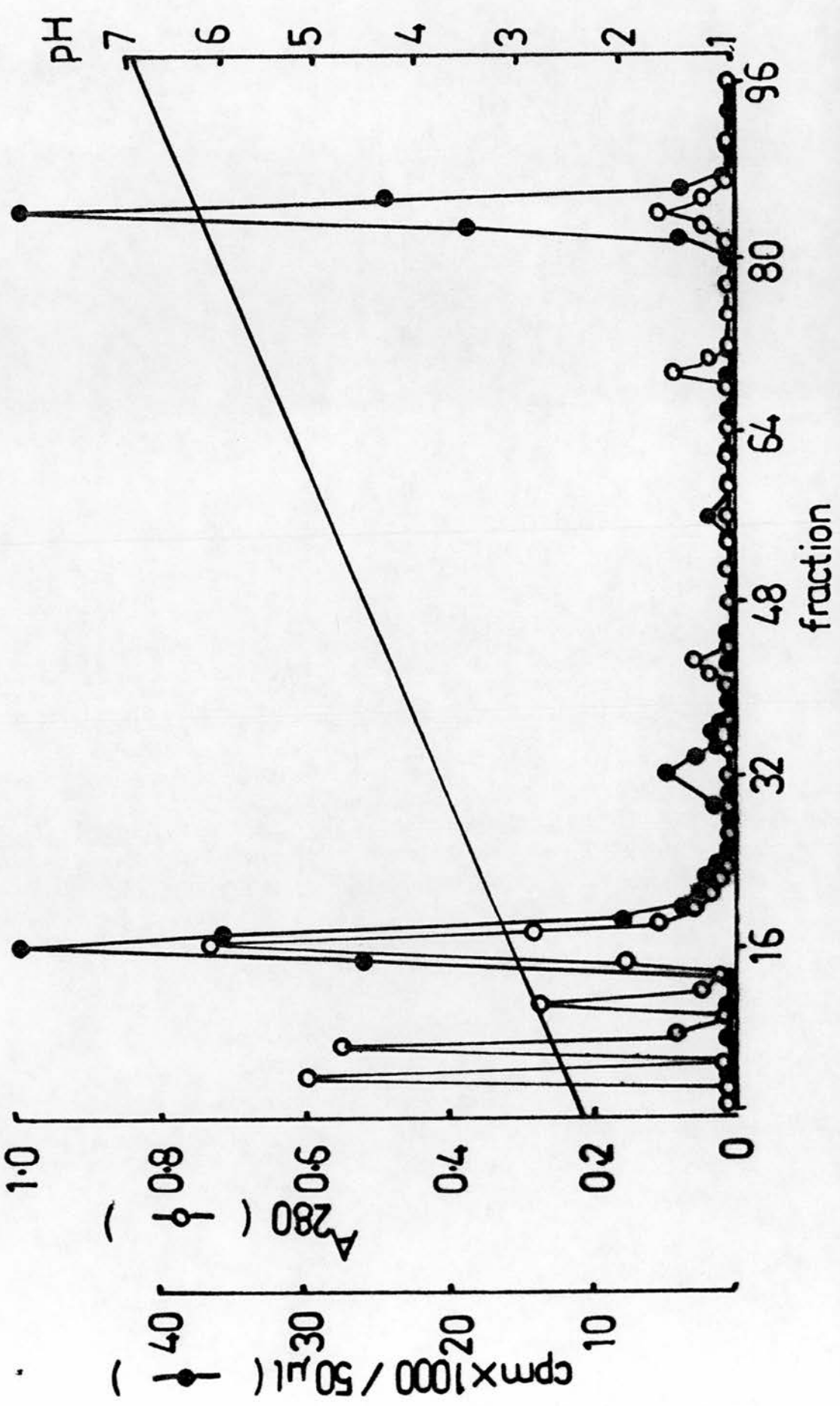
This major peak of radiolabelled peptide was freeze-dried and taken up in 50mM ammonium formate, pH3.0 before being applied to a column of Dowex AG50W-X8 (1cm x 16cm) anion exchange resin equilibrated in the same buffer. The column was washed with a stepwise pH gradient of 50mM ammonium formate pH3.0 - pH5.0 then 50mM sodium acetate pH5.1 - pH7.0 as described in fig. 6-7. One main peak of radiolabelled peptide eluted at pH3.0 - 3.5. During one preparation an additional peak of radioactivity eluted at pH6.0. Analysis of this minor peak is described in section 6.2.7.

6.2.7 Spectral analysis of the photolabelled peptides

After the main radiolabelled peak described above had been freeze-dried and taken up in water, its spectral properties between the wavelengths 240 - 360nm were recorded. As shown in fig. 6-8 there was a well-defined absorbance maximum at 260nm consistent with the notion that an aromatic moiety of NAD^+ was present. Reaction of the sample with potassium cyanide as described in section 2.11 produced a new absorbance peak at 340nm. When a solution of NAD^+ of comparable initial absorbance at 260nm was treated in the same way, an identical peak of absorbance at 340nm was produced. Spectral curves for the oxidised and cyanide addition product forms of NAD^+ and various derivatives thereof have been published (Sigel *et al* ., 1959). When compared to the curves of NAD^+, 3-acetyl pyridine adenine dinucleotide, 3-pyridine aldehydeNAD^+, deaminoNAD^+, 3-acetyl pyridine deamino NAD^+, 3-pyridine aldehyde deaminoNAD^+ and thioNAD^+, the absorbance maxima at 260nm and 340nm of the radiolabelled sample cyanide complex and the shape of the spectral

fig. 6-7 Ion exchange chromatography of tryptic peptides from radiolabelled A_1 chain

Fractions making up the main peak of radiolabelled peptide in fig. 5-6 were pooled, freeze-dried and applied to a column of Dowex AG50W-X8 in 50mM ammonium formate, pH3.0. The column was washed with ammonium formate buffer of increasing pH from 3.0 to 5.0 then with 50mM sodium acetate from pH5.1 to 7.0. The column was run at 1ml/min. and fractions of 1ml collected and assayed for radioactivity and for absorbance at 280nm. The sloping line indicates the pH gradient.


pH
7
6
5
4
3
2
1
96
80
64
48
32
16
0
fraction
1·0
0·8
0·6
0·4
0·2
0
A_{280} ( )
40
30
20
10
cpm x 1000 / 50 µl ( )

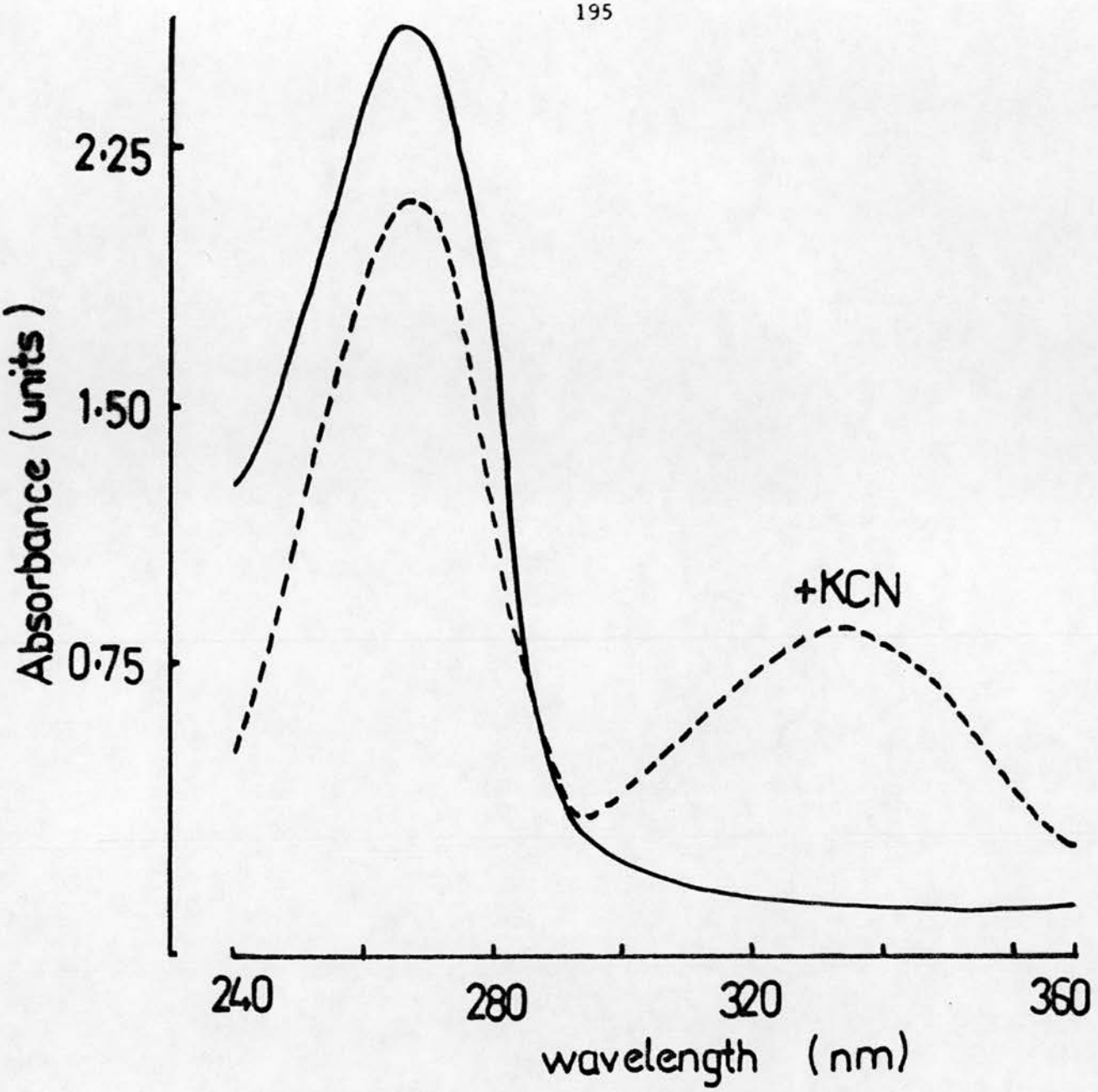


fig. 6-8 **Absorbance of the major radiolabelled peptide in the presence and absence of potassium cyanide**

Spectra were recorded using a Carey spectrophotometer as described in materials and methods

curve most closely resembled those of NAD^+ itself. The cyanide peak (340nm) of the sample was similar to that of NMN but the spectra differed in other ways. NMN has a two-component peak of absorbance at 274nm and 256nm which dissappears completely on reaction with cyanide. This suggests that the NAD^+ incorporated into the peptide has not been converted into any of the above derivatives. Most of these derivatives can be synthesised either enzymically, whereby NAD'ase enzymes are used to catalyse the exchange of nicotinamide for various derivatives or by chemical modification such as the deamination of the adenine moiety. Presumably, photoexcitation of the NAD^+ has not caused any of these modifications.

In contrast, the minor radio-labelled peak described in section 6.2.6 showed no reaction at all with potassium cyanide (fig. 6-9). Its spectrum showed a peak with an absorbance maximum at 264nm and a shoulder at 256nm. The absorbance maximum of nicotinamide is at 264nm, and the spectrum described is similar but not identical to the spectrum of NMN which has a maximum at 266nm with shoulders at 274nm and 256nm. However, reaction of NMN with cyanide produces a large absorbance maximum at 325nm (P.L. Biochemicals information sheet 6, 1973). Cyanide forms an addition product with the pyridine ring of N-substituted nicotinamides (Colowick and Womack, 1969). The incorporated NAD^+ or derivative in this sample has presumably become linked to the toxin in such a way as to prevent the reaction of cyanide with the nicotinamide ring, as at least part of the nicotinamide ring (carbon 4) containing the tritium label must be

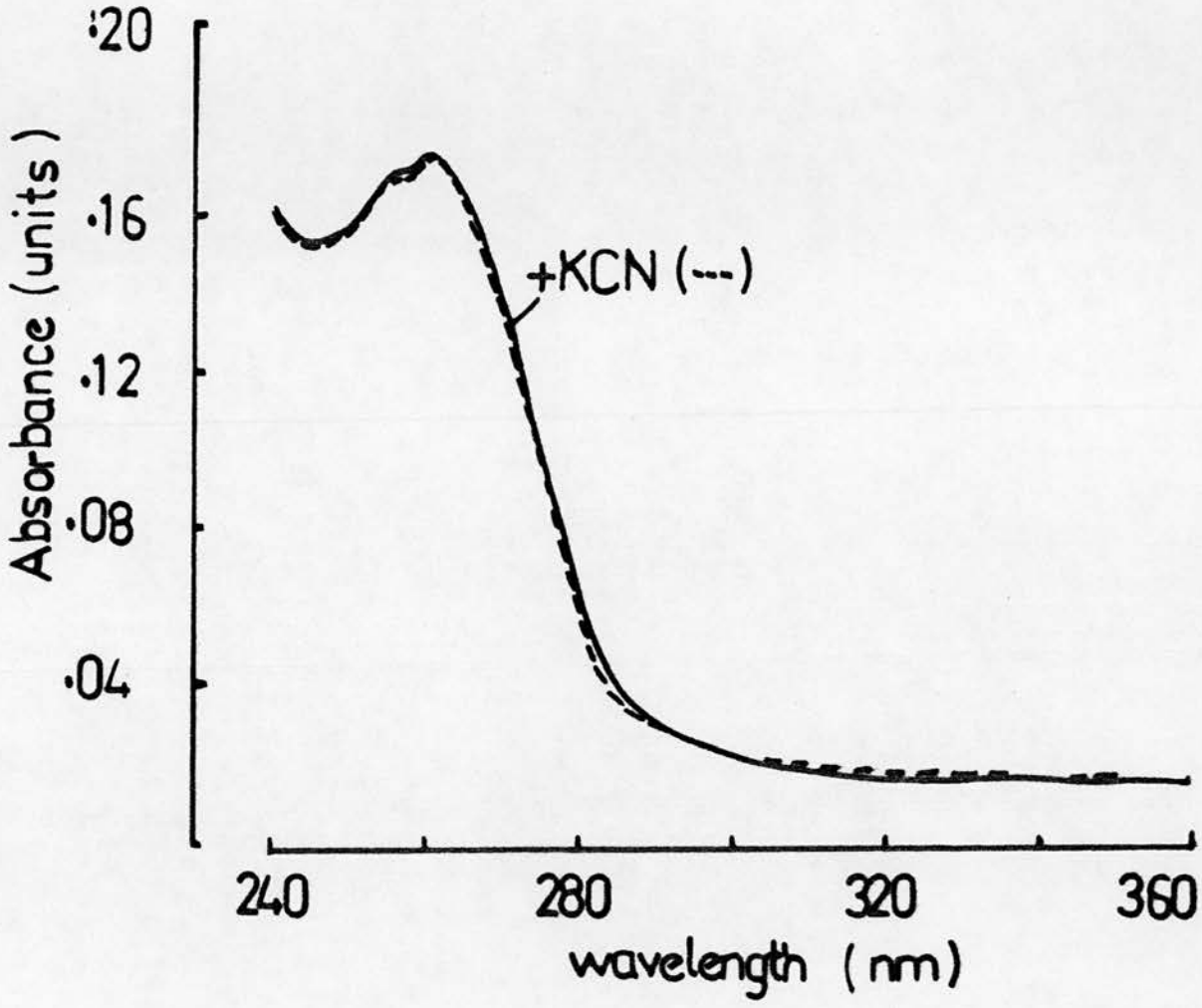


fig. 6-9 Absorbance of the minor radiolabelled peptide in the presence and absence of potassium cyanide

present. Two distinct NAD^+-toxin complexes have therefore been isolated.

6.2.8 Amino acids analysis

Amino acid analysis was performed in an attempt to identify the radiolabelled peptide by comparison with the known amino acid sequence of the A_1 chain. Initial attempts were made using an LKB 4400 analyser in the Department of Biochemistry, University of Edinburgh with the help of Mr. Andy Cronshaw. This method employs ion exchange chromatography followed by post-column derivatization with ninhydrin in a low volume, high temperature reaction coil. Upon reaction with ninhydrin, most amino acids produce a complex which absorbs strongly at 570nm; however, the imino acid proline forms a complex with different absorbance characteristics which can be detected if a dual channel detector, with the second channel set at 440nm, is used.

The peptide samples (both 'major' and 'minor' peaks mentioned above) were hydrolysed in evacuated tubes with 6 M HCl at 110°C for 22h. When hydrolysis was complete the tubes were opened and HCl removed under vacuum. Samples were injected into the analyser in a pH2.2 buffer (Davies and Thomas, 1973; Moore and Stein, 1963) and separation of the protein hydrolysate achieved using a system of three citrate buffers (Condon *et al.*, 1984). Chromatograms were produced for both the major and minor peak samples. The chromatograms show good resolution of all amino acids but with some disturbance in the 570nm baseline in the basic region of the chromatogram. The results for both samples suggest that both "major" and "minor" peaks represent the same peptide. Both samples

contain large amounts of glycine, the major peptide sample in particular. As the ratio of glycine to other amino acids in this sample does not correspond to any tryptic peptide within the A_1 chain (Duffy *et al.*, 1985) it seems likely to be a contaminant. Glycine, in common with serine and certain other polar amino acids is the most common environmental contaminant in samples for amino acid analysis, possibly from atmospheric dust (Cohen *et al.*, 1986). As shown in Table 6-1 which summarises the results, the ninhydrin method detects lysine in the samples, but no arginine. A small peak has been assigned to arginine in the original traces. However, the arginine peak was incompletely resolved from the large ammonia peak and occurred at a region of disturbed baseline. The automatic integrator used with this analyser may have overestimated the presence of arginine, likewise of histidine which also appears at a region of disturbed baseline, caused by a change in buffer.

The major sample peak (none of the minor peak sample remained) was analysed further using an alternative method of analysis. This was kindly performed by Mr. Ian Davidson in the Department of Biochemistry, University of Aberdeen. The method is based on a gaseous acid hydrolysis of the sample followed by the formation of a phenyl-thiocarbamyl (PTC) derivative of the amino acids by derivatization with phenyl isothiocyanate (PITC) (Bidlingmeyer*et al.*, 1984; Koop *et al.*, 1982). The derivatized amino acids are then separated by reverse phase hplc, followed by ultraviolet detection at 254nm. This method can detect as little as one picomole of each amino acid under certain conditions and can provide extremely rapid analysis. A chromatogram of the radiolabelled sample is shown in

fig. 6-10 and the results summarised in table 6-1. Again, the presence of lysine is indicated with this time a complete absence of arginine. There is a large peak corresponding to glycine which again may be elevated by atmospheric contamination. Two extremely large peaks have not been assigned to any amino acid. They may represent hydrolysis breakdown products of NAD^+ which would absorb strongly around 260nm and would thus be visible in the 254nm trace. Such NAD^+ derivatives would be unlikely to react with ninhydrin and would not be visible at 570nm.

The fact that both analysis methods suggest the presence of lysine simplifies the search for the tryptic peptide as the A_1 chain contains only two lysine residues, at positions 4 and 17, close to the amino-terminus. The sequence of the first 20 residues of the A_1 chain is shown below table 6-1. If the peptide contains lysine then it must be either Asn·Asp·Asp·Lys or Pro·Pro·Asp·Glu·ile·lys. An empirical formula calculated from the PTC results fits exactly with the first peptide (if the glycine is ignored completely and the unknown peaks are assumed to be NAD^+ derivatives). Asparagine and aspartate cannot be distinguished following acid hydrolysis. An empirical formula from the ninhydrin results does not fit so well with the first peptide, but neither does it fit with the second

fig. 6-10 Amino acid analysis of the major radiolabelled peptide

The analysis used reverse phase hplc on a "μBONDAPAK™" C_{18} column and an acetate/acetonitrile gradient system. The gradient was 100% to 0% acetate, 0% to 100% acetonitrile over 12 min. at a flow rate of 1.0ml/min. followed by an 8.0 min. wash with 100% acetate at flow rate 1.5ml/min. The chart speed was 2cm/min. with detection at 254nm.

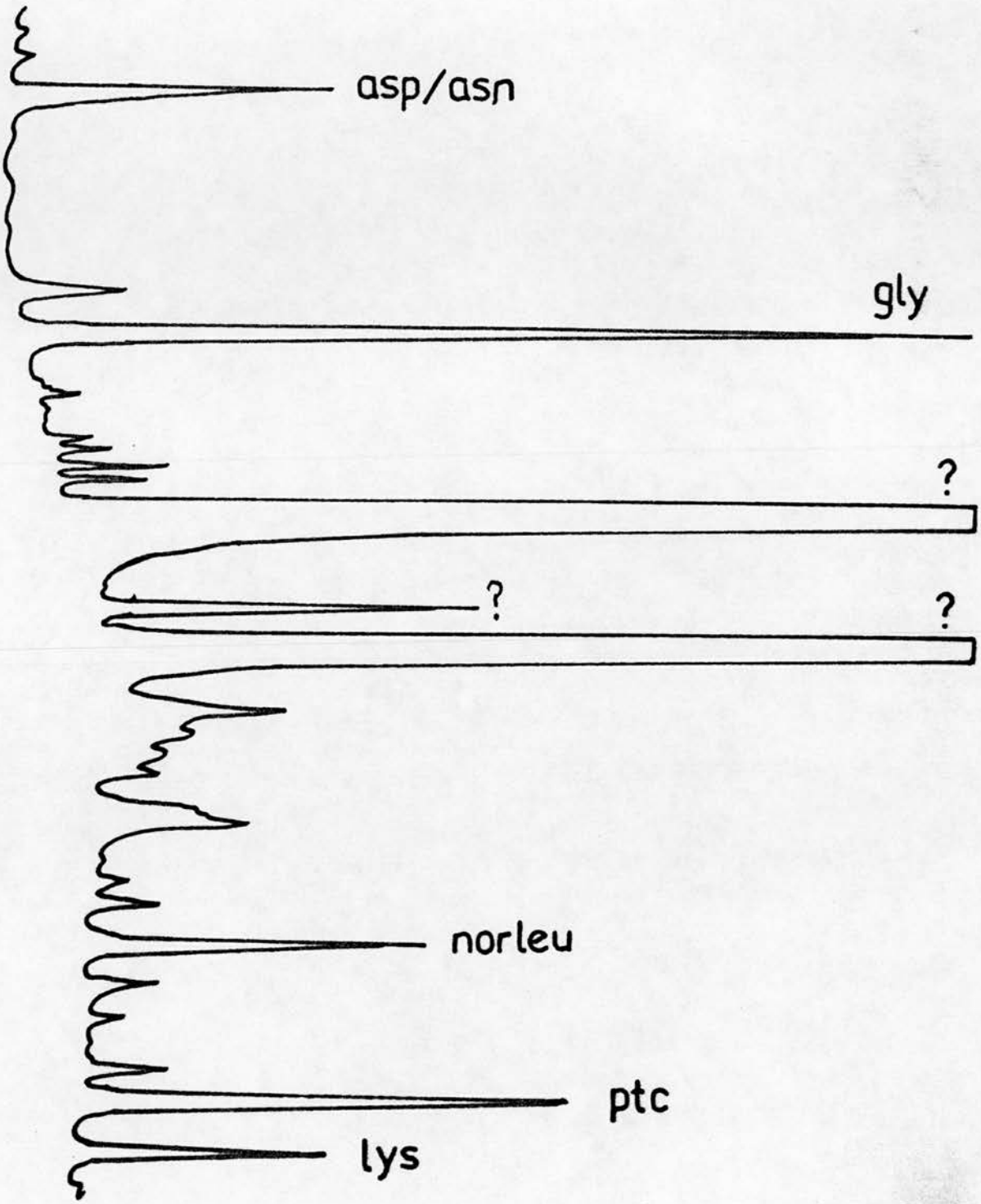

asp/asn
gly
?
?
?
norleu
ptc
lys

table 6-1 Amino acid analysis of the major radiolabelled peptide: results from both ninhydrin and PTC analyses

amino acid	ninhydrin		PTC	
	nMoles detected	empirical formula	nMoles detected	empirical formula
Asp	5.90	2	1.47	3
Thr				
Ser				
Glu	6.24	2		
Pro				
Gly	6.82*	2	3.34	6
Ala	4.74	2		
Cys				
Val				
Met				
Ile				
Leu	2.32	1		
Nor				
Tyr				
Phe				
His	2.32	1		
Lys	2.81	1	0.58	1
Arg				

* in one expenment, Gly was offscale.

NH_2 - ASN - ASP - ASP - LYS - LEU - TYR - ARG - ALA - ASP - SER -
1 1 3 4 5 6 7 8 9 10

ARG - PRO - PRO - ASP - GLU - ILE - LYS - GLN - SER - GLY
11 12 13 14 15 16 17 18 19 20

peptide, even after allowance has been taken for the poor reaction of proline with ninhydrin. As mentioned, polar amino acids such as serine, threonine, glycine and alanine are the most common contaminants in analyses of this sort. Contamination through handling the tubes with the fingertips, from dust or from sample buffers may have led to the low levels of some polar amino acids in the ninhydrin trace.

6.2.9 Amino acid sequencing

The sample of radiolabelled peptide was then subjected to amino acid sequencing which was kindly performed by Mr. Bryan Dunbar in the Department of Biochemistry, University of Aberdeen. Sequencing was done automatically with an Applied Biosystems gas phase sequencer, operated as described in Materials and Methods. In the gas phase machine, the sample is contained on the surface of a thin disc of glass filter mounted inside a glass column. Polybrene, a polymeric quaternary ammonium salt that adheres to both glass and peptide is added to retain the sample. The Edman chemicals (Edman, 1956; Edman and Begg, 1967) are then added as vapours, e.g. in argon. In this case polybrene (1mg) was added to the sintered glass fibre sample disc and subjected to a three hour cycle of washes before the sample of radiolabelled peptide (approximately 600nmol) was introduced. The sequencer automatically converted thiazotinones to thiohydantoin derivatives by reaction with trifluoroacetic acid, and then delivered each sample into a Waters low volume insert vial. The samples were dried in a Speedvac centrifuge for 15 min. at room temperature. Acetate buffer, pH5.0 (15µl) and acetonitrile (15µl) were then added at least 15 min. before hplc analysis.

The phenyl thiohydantoin derivatives were identified by hplc on Apex Cyano columns (0.4 x 25cm) with an acetate buffer pH5.0/ acetonitrile gradient system and a 254nm detector. Repetitive yields were calculated by linear regression analysis of the yields at each cycle, omitting PTH-Ser, PTH-Thr, PTH-ile and PTH-Scarboxymethyl Cys all of which had low yields. When the results for an analysis of radiolabelled peptide were examined and compared to a standard run, no residues other than the internal standard (0.5nmol PTH norleucine) were present in any of the first six cycles. The same result was obtained when a larger volume of sample (25µl) was injected). This indicates either that there is no peptide in the sample (but this is not the case as shown by amino acid analysis) or the amino-terminal residue is blocked from reacting with phenylisothio cyanate. Certain conditions can hinder the reaction of some amino acids with phenylisothio cyanate. If the peptide contains a large number of aspartate residues, acid catalysed cleavage can obscure amino acid components by causing a high background. Residues linked to those with bulky side chains may be incompletely cleaved suggesting that if the NAD^+ had become incorporated anywhere near the amino terminus it could interfere with cleavage. The presence of high concentrations of DTT in sample buffers (there was 20mM DTT in the original buffer) can obscure hplc peaks of aspartate (Hunkapiller and Hood, 1978; Hewick *et al.*, 1981; Wittman-Liebold, 1982). In conclusion then, no sequence could be determined by this method for the radiolabelled peptide as the N-terminal residue did not react with the Edman chemicals, either the irradiation procedure had modified the peptide and/or the

incorporation of ^{3}H-NAD^{+} was interfering with the reaction.

6.2.10 Dansyl chloride end group analysis

This analysis was done in the Department of Biochemistry of the Dick Vet School, Edinburgh.

Amino-terminal analysis was performed using dansyl chloride, with chromatography of the dansyl derivatives on polyamide thin layers as described in Materials and Methods. Approximately 10 nMoles of the radiolabelled peptide was treated with dansyl chloride as described and then hydrolysed in an evacuated-tube with 6M HCl at 110°C for 20h. After this time the tubes were opened and HCl removed under vacuum over NaOH pellets. The hydrolysed sample was chromatographed as described and visualised under ultraviolet light at 365nm. The developed plate and its corresponding standard are shown in fig. 6-11. The only spots visible were those corresponding to Dns-NH_3 and Dns-OH, with nothing which might represent a dansyl-end group derivative. It was previously shown (Chapter Three) that the end group of the A_1 chain could be identified as asparagine by this method, indicating that the end residue is not normally blocked from reaction with dansyl chloride. A possible effect of irradiation and photoaffinity labelling of the peptide could have been to cause some alteration in the end residue to block its subsequent reaction with dansyl chloride. The NAD^{+} could have become photoincorporated into the amino-terminal asparagine itself. Photoincorporation has previously been reported to hinder the Edman reaction (Lifter *et al.*, 1974). The insertion of a nitrene group into the αcarbon of tyrosine 88 in myeloma protein 460 apparently hindered Edman degradation. The direct effects of irradiation upon peptide bonds

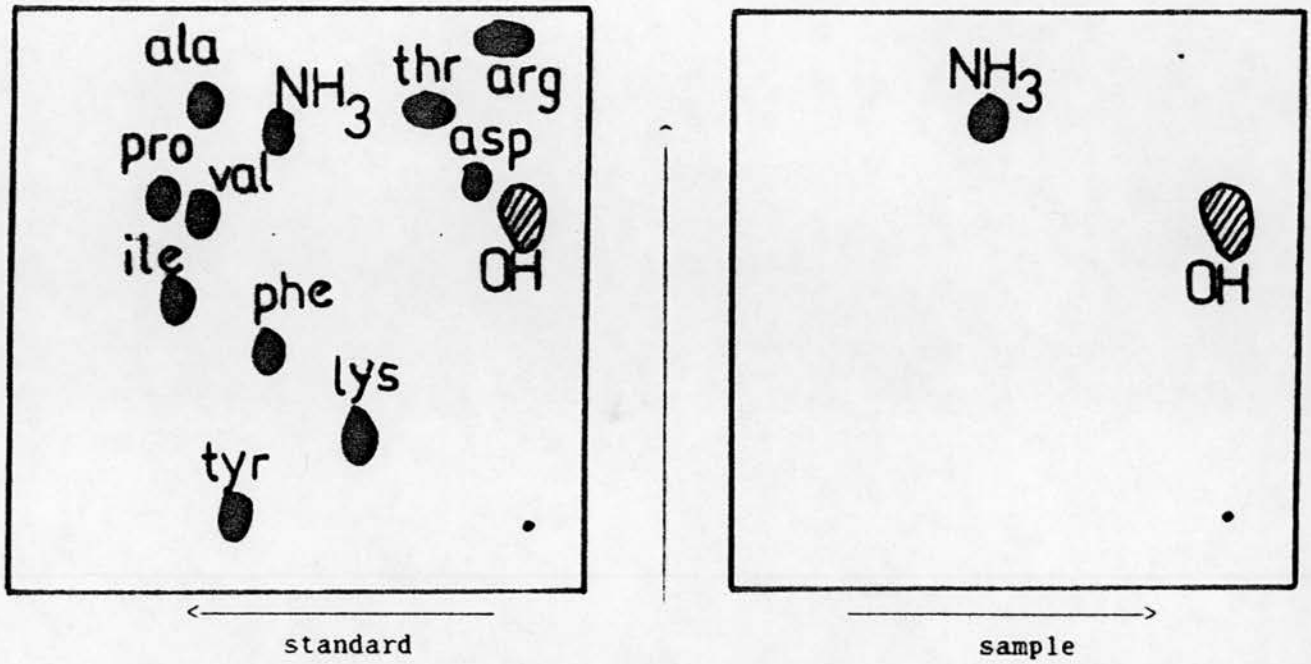


fig. 6-11 **Thin layer chromatography of dansyl-amino acid derivatives on polyamide sheets**

The solvent system used is described in section 2.18

may also prevent Edman degradation. The same authors report that the conversion of an α-peptide bond between asparagine and glycine into a β-aspartyl linkage, presumably the result of irradiation, left the peptide unreactive. Something similar may have occurred here. On the other hand, the purification procedure itself could have caused some unknown alteration in the peptide's structure.

No results concerning the nature of the peptide were obtained using this method. The N-terminal residue of the peptide had once again failed to react, this time with dansyl chloride.

6.2.11 Fast-atom-bombardment mss spectrometry

This work was kindly performed by Mr. Alan Taylor of the Department of Chemistry, University of Edinburgh. Fast-atom-bombardment (FAB) mass spectra were recorded using argon gas on a MS5OTC model spectrometer with Data General software models DG30 and DS90. Calibration was with caesium iodide and a copper or stainless steel tip was used for the FAB probe.

Portions of freeze-dried sample were dissolved in various solvents listed in table 6-2 and applied to the tip of the FAB probe. In FAB mass spectrometry the sample is exposed to an ionising beam which gently fragments the sample. The fragments pass over a magnet which deflects them to land at a position dependant upon their mass/charge ratio.

A total of 84 scans were run using the solvents listed, of which PEG 200 and tetraglyme yielded the best results. Thioglycerol at first appeared to be suitable but a small solvent peak was later found to be interfering with the main sample peak at 1147 (fig. 6-12). The other solvents used were found to contain no

contaminating peaks in this area. A species corresponding to molecular weight 1147 was found to be present to a varying degree in all the scans. There were also signs of apparent double-charged forms of this peptide at positions 530-560. These occur when the species accepts more than one charge. As shown in fig. 6-12 the largest of these peaks corresponded to mass charge 555.

These results show that the sample of radiolabelled peptide contains a species of molecular weight 1147. This corresponds to the molecular weight of the tetrapeptide asn·asp·asp·lys plus [4-^{3}H]NAD^{+}. It is not possible from these results to say to which residue the NAD^{+} is bound or what the chemistry of binding is. The number of multiple-charged species and the relatively low level of the 1147 species suggest that the sample has by this time suffered some level of degradation and that its structure contains a number of charged groups.

table 6-2 Solvents used for FAB mass spectrometry analysis of the radiolabelled peptide

solvent	number of scans
thioglycerol	15
glycerol+oxaloacetate	10
sulpholane	8
chloroform	5
dibutylphathaldehyde	14
benzyl benzoate	3
PEG 200 (polyethylene glycol)	16
PEG 200/dimethylformalate	3
dimethylformalate/glycerol	5
tetraglyme (tetramethylene glycol dimethyl ether)	5

fig. 6-12 Fast atom bombardment mass spectra of the radiolabelled peptide sample

(a) The solvent used was thioglycerol. In this trace only the main peaks are shown. The peak at 1147 cannot be resolved from a solvent peak at the same position.

(b) The solvent was dibutyl phathaldehyde. The large peak at 555 and the smaller peaks at 539 and 569 may correspond to double-charged species, the variation in position arising from the loss of one or more fragments during the FAB process.

fig. 6-12(a)

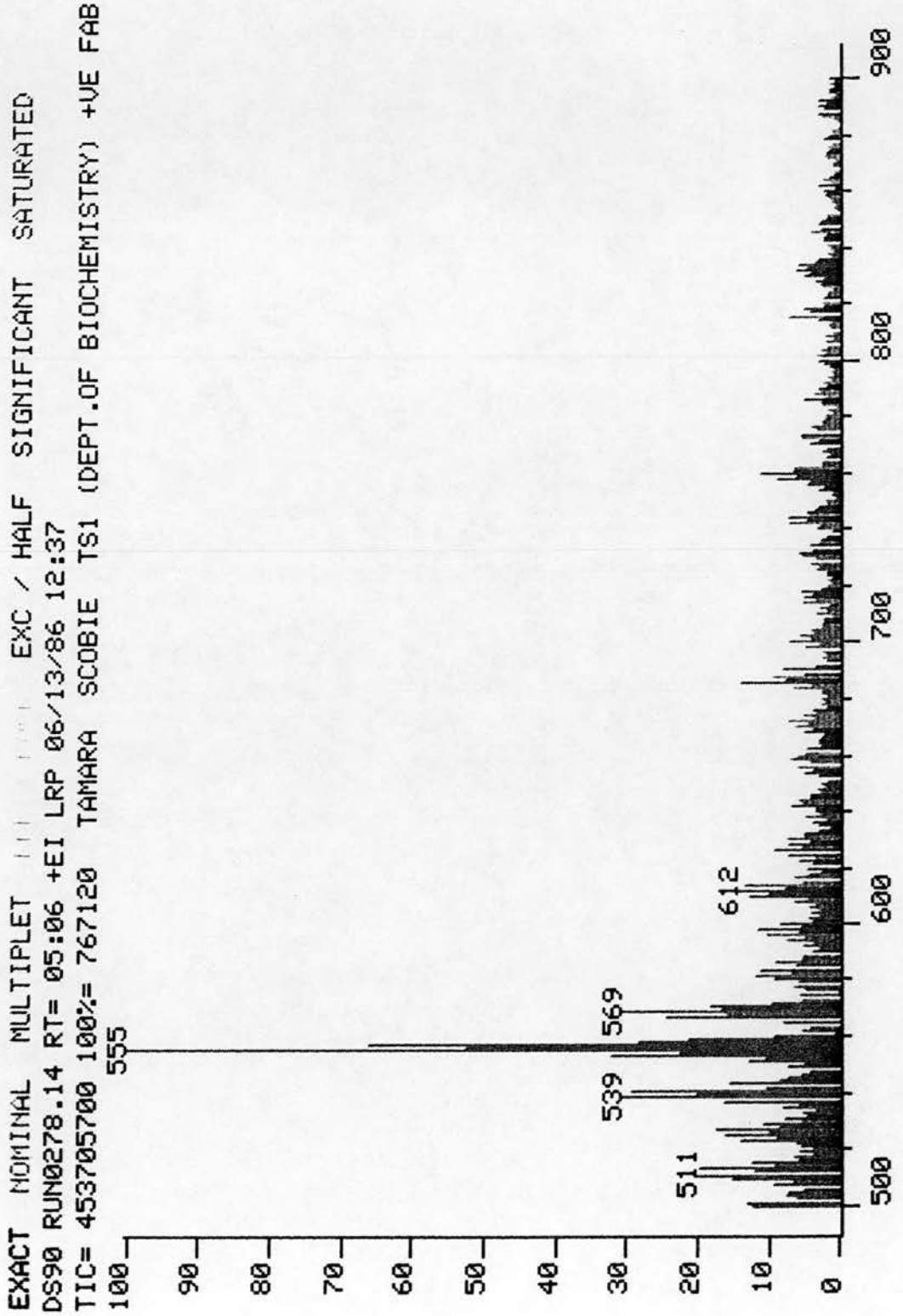
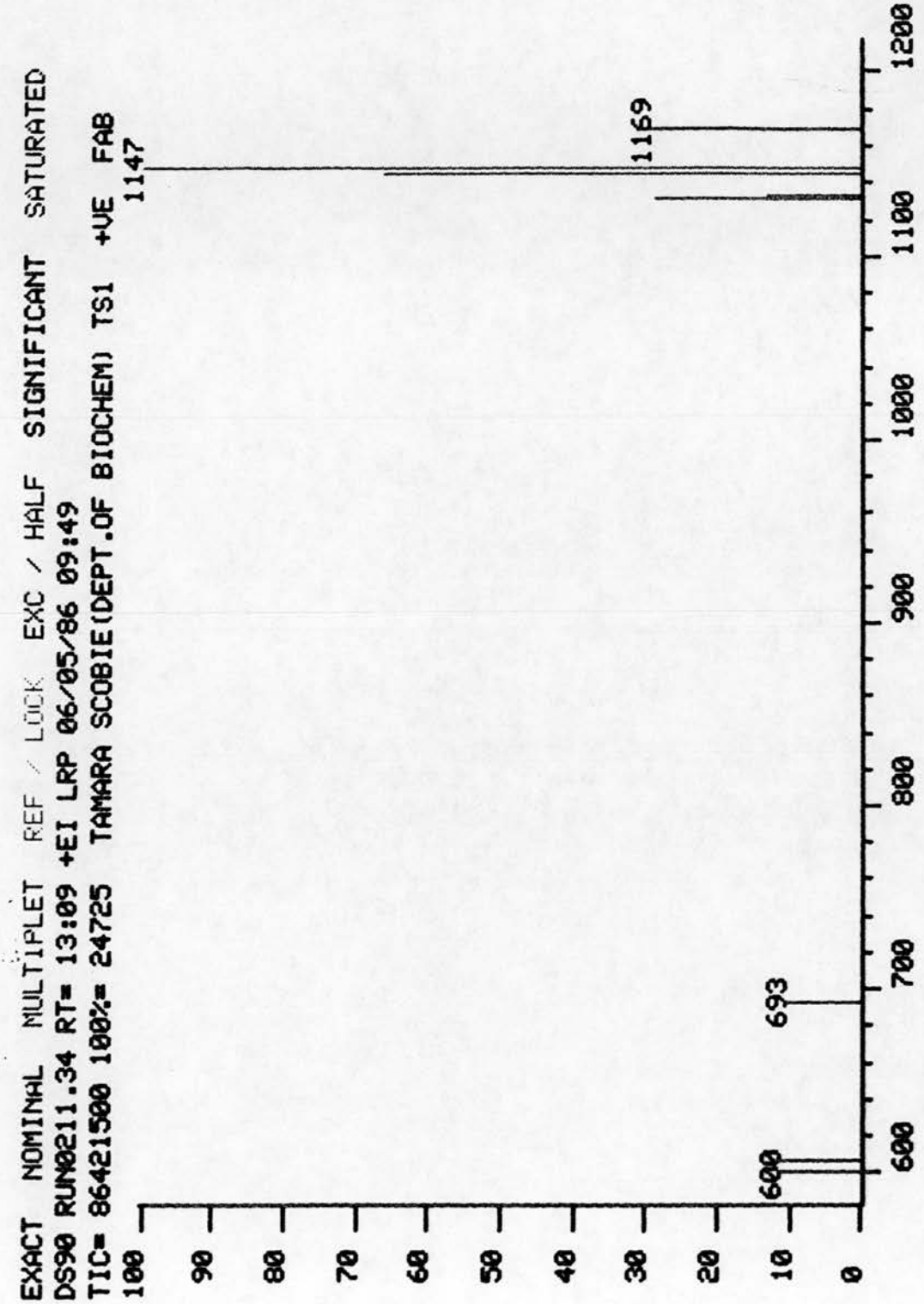

EXACT NOMINAL MULTIPLET EXC / HALF SIGNIFICANT SATURATED
DS90 RUN0278.14 RT= 05:06 +EI LRP 06/13/86 12:37
TIC= 453705700 100%= 767120 TAMARA SCOBIE TS1 (DEPT.OF BIOCHEMISTRY) +VE FAB
100
90
80
70
60
50
40
30
20
10
0
555
569
539
511
612
500
600
700
800
900

fig. 6-12( )


EXACT NOMINAL MULTIPLET REF / LOCK EXC / HALF SIGNIFICANT SATURATED
DS90 RUN0211.34 RT= 13:09 +EI LRP 06/05/86 09:49
TIC= 86421500 100%= 24725 TAMARA SCOBIE(DEPT.OF BIOCHEM) TS1 +VE FAB
1147
1169
693
600
100
90
80
70
60
50
40
30
20
10
0
600
700
800
900
1000
1100
1200

6.3 Discussion

6.3.1 Nature of the photo induced incorporation of NAD^+

The results presented here show that irradiation of mixtures of cholera toxin and NAD^+ with short wave ultraviolet light leads to the covalent cross-linking of NAD^+ to a specific region of the toxin's A_1 chain. Incorporation was saturable with between 0.05 and 0.20 moles of NAD^+ incorporated per mole of toxin. The protein-NAD^+ linkage was stable in 12% TCA, 5% formic acid, 6M urea, 50mM tris-HCl, 0.1% SDS, methanol and acetic acid. This strongly suggests that the linkage is covalent. It was not susceptible to cleavage with trypsin. The similarity in the incorporation of adenine or nicotinamide radiolabelled NAD^+ together with the spectrum obtained on reaction of a purified radiolabelled peptide with potassium cyanide both suggest that most, if not all of the NAD^+ molecule had been incorporated into the main peak of radiolabelled peptide. This last point contrasts with results reported for diphtheria toxin (Carroll and Collier, 1984; Carroll *et al.*, 1985). Photoinduced incorporation of NAD^+ was much more efficient with nicotinamide-labelled NAD^+ than it was with NAD^+ labelled in other portions of the molecule and the photolabelled product was eventually identified to be glutamate with the nicotinamide moiety of NAD^+ attached. The ADP-ribose group was not present and had presumably been cleaved either by the effects of irradiation or by the intrinsic NAD'ase activity of diphtheria toxin. The apparent stability of adenine-radiolabelled NAD^+ (at least in the main peptide) when attached to cholera toxin could reflect a lower level of NAD'ase activity, a lowered susceptibility to photoinduced cleavage of the nicotinamide-

ribose bond, or it could indicate a difference in the nature of the NAD^+-toxin attachment.

6.3.2 Nature of the radiolabelled peptide

Gel filtration and ion exchange chromatography allowed the isolation of a discrete peak of radiolabelled peptide. The apparent level of incorporation of NAD^+ for this peptide was approximately 0.7 moles NAD^+ per mole peptide. It is not possible to say from the results whether one or more molecules of NAD^+ were incorporated or whether the NAD^+ was linked to one particular residue within the peptide. However, preliminary results using fast-atom-bombardment mass spectrometry indicate the presence of a major species of molecular weight 1147. This is very close to the molecular weight of 1155 obtained by adding the weights of one NAD^+ and the tetra peptide Asn·Asp·Asp·Lys. The nature of this apparently pure radiolabelled peptide was investigated using a number of techniques. Amino acid analysis allowed an estimate of the amount of peptide present. As shown in table 6-1 the results obtained from a ninhydrin ion-exchange system analyser and a phenyl thio carbamyl (PTC) derivative hplc separation analyser were not identical. However, they both indicate that the peptide, whatever it is, is small. It contains almost exclusively polar amino acids such as aspartate, glutamate, lysine with [illegible] leucine and alanine and a large amount of glycine. The PTC analysis which was five times more sensitive than the ninhydrin trace suggested the presence of even fewer components; aspartate, lysine and again a large amount of glycine. If the glycine is presumed to be a contaminant, then this analysis fits very well with one of the two lysine-containing

tryptic peptides in the A_1 chain corresponding to residues 1-4; Asn·Asp·Asp·Lys. The ninhydrin analysis does not discount this peptide as a possibility. It suggests the presence of aspartate and lysine in an approximate 2:1 ratio. The other amino acids present are all common environmental contaminants (Hunkapilla and Hood, 1978) although serine and threonine which are also reportedly two of the most common contaminants are not present. If the tetra peptide Asn·Asp·Asp·Lys is a possible candidate then there is no indication to which residue(s) the NAD^+ label has become attached. Such a small peptide might not have been expected to elute in the position it did following gel filtration chromatography (fig. 6-6). However the tryptic peptides loaded onto this column may have clumped together and been poorly resolved. The electrophoretic mobility and other physical properties important in the separation of such a small peptide may have been changed by the covalent binding of the ligand (Bayley and Knowles, 1977). A number of investigators have observed that photolabelled proteins and peptides do not always comigrate exactly with their unlabelled counterparts (Cooperman *et al.*, 1975; Girshovich *et al.*, 1974; Hsiung *et al.*, 1974).

Amino acid sequencing and end group analysis using dansyl chloride, both techniques which might be expected to give more detailed information, if not proof of the exact nature of this peptide, both failed to provide any information concerning the nature of the amino acids present as the radiolabelled peptide was apparently blocked from reaction with the chemicals used. The lack of any reaction with dansyl chloride does suggest that the peptide sample is pure as free amino acids might be expected to react with the dansyl chloride

themselves. Contamination of the samples with glycine as evident in amino acid analysis, could have occurred at any stage in their preparation for the analysis. The possibility of photoaffinity labelling interfering with the Edman degradation was mentioned in section 5.2.10.

6.3.3 The minor radiolabelled peptide

Followng ion-exchange chromatography of one batch of radiolabelled toxin (fig. 6-6), a "minor" peak of radiolabelled material was eluted at higher pH (pH 6.0 - 6.5). This peak was only present once out of a total of three radiolabelling experiments (all of which produced an identical 'major' peak), and very little was available for analysis. However amino acid analysis using the ninhydrin method showed that the major and minor peptides had the same amino acid composition. A difference was noted in their absorbance spectra and in their ability to react with cyanide. The absorbance spectrum of the minor peak resembled that of NMN in having a maximum at 264nm with a shoulder at 256nm. Unlike NMN, there was no reaction at all with cyanide although the nicotinamide ring containing the tritium group must have been present. The elution of the major and minor peaks at different pH values suggests that they may have a different net charge, presumably caused by a difference in the incorporation of the NAD^+. This could highlight the indiscriminate nature of the photolabelling procedure and a possible randomness of incorporation (e.g. see Fleet *et al.*, 1972; Fisher and Press, 1974), or it could mean that the NAD^+ is attached in the same

way but, in the case of the minor peptide, has become degraded or altered following irradiation and purification.

6.3.4 The amino terminal peptide as a component of the active site

One interpretation of these results is that the [4-^{3}H] NAD^+ has become covalently linked to an unspecified amino acid within the first four residues of cholera toxin's A_1 chain (for the complete amino acid sequence of the A subunit of cholera toxin, see Appendix C). How does this result compare with what is known about the structure of the A_1 chain? The complete amino acid and nucleotide sequences of both subunits of cholera toxin are known, and have been compared to those of the highly homologous *E.coli* LT (see for instance Dykes *et al.*, 1985; Spicer and Noble, 1982; Lockman *et al.*, 1984). Certain regions of completely conserved nucleotide sequence have been related to domains of the two proteins important for activity. Ideas about the secondary structure of the A_1 chain have been based on predictions from analysis of the primary structure, as no crystal suitable for detailed study of the structure have yet been reported (see Sigler *et al.*, 1977 for preliminary crystallographic results). Further ideas on the regions of the toxin responsible for activity have come from activity studies with proteolytic fragments (Matus *et al.*, 1976; Lai *et al.*, 1981) and monoclonal antibodies directed at particular areas of the toxin (Kurosky *et al.*, 1982a). Results from these different approaches can be summarised as follows: Yamamoto *et al.*, (1984) showed that the amino terminal residues of cholera toxin and of *E.coli* LT had been very closely conserved, more so than the carboxyl-terminal half of the toxins. In fact there were several regions of 100%

homology including residues 3 - 17, 29 - 54, 81 - 102 and 110 - 130. Comparison of the first four residues of each toxin is shown below.

Asn·Gly·Asp·Lys
Asn·Asp·Asp·Lys
1 2 3 4

The top sequence is for *E.coli* LT (Yamamoto *et al.*, 1984, although several other authors suggest that residue 4 in LT may be arginine (Dykes *et al.*, 1985; Spicer and Noble, 1982). These regions have most likely been conserved between the two proteins. It seems unlikely that they would have been so strongly conserved if they were not involved in some way in the toxin's activity. (There is abrupt and complete divergence between the cholera toxin and *E.coli* LT nucleotide sequences outside the structural genes (Lockman *et al.*, 1984)). A secondary structure prediction based on this sequence (Yamamoto *et al.*, 1984) suggests that residues 3 - 17, being highly charged, may be close to the surface of the protein and may form part of the active site. However, the fact that monoclonal antibodies directed towards residues 95 - 140 of the A_1 chain could inhibit ADP-ribosylation (Kurasky *et al.*, 1982a) suggests that this region of the toxin may also be important for activity and may be looped out on the surface of the protein (Duffy *et al.*, 1985). Additional evidence that this region may be important in the catalytic activity of the toxin was suggested by the discovery of an auto-ADP-ribosylation site at arginine 146 of the A_1 chain (Lai *et al.*, 1983). Limited tryptic digestion of the A_1 chain led to reports of a modified core peptide of molecular weight 12,500 derived from residues 47 - 156 of the A_1 chain, which apparently

retained almost 50% of the catalytic activity of whole A_1 chain (Lai *et al.*, 1981). This suggested that the central residues of the A_1 chain contained the catalytic site, but the finding that this core peptide lost its activity following purification implied that an active conformation had been lost during purification or that other parts of the A_1 chain were also involved in the active site. In fact the authors never showed that the activity they detected was a property of the 12,500 peptide; rather, activity was present only in a mixture of peptides formed by limited tryptic digestion of the A_1 chain. Just about any portion of the A_1 chain could have been involved.

There have even been tentative predictions, based on primary sequence, that cholera toxin shares with diphtheria toxin a βαβα structure similar to that reported for lactate dehydrogenase (Rossman *et al.*, 1974). As mentioned in Chapter Five, direct evidence from X-ray crystallographic studies of exotoxin A (Allured *et al.*, 1986) failed to detect any such structure and as diphtheria toxin and exotoxin A are reportedly so similar (e.g. Chung and Collier, 1977b; Carroll and Collier, 1984) it is unlikely that such a region of structure exists in either diphtheria toxin or cholera toxin. The evidence so far loosely implicates a central region of the toxin as a likely location for the catalytic site but with definite involvement from other parts of the toxin. As mentioned in section 6.1, Xia *et al.*, (1984) suggests that since cleavage of the disulphide bond between A_1 and A_2 is required for activity, the carboxyl terminal of the A_1 chain close to the cysteine at 188 may be involved in the active site. Reduction might expose this region

to the substrates and a change in conformation in this area has been noted following reduction (de Wolf *et al.*, 1985). However, both amino and carboxyl terminals are predicted to lie closely together in the native protein (Duffy *et al.*, 1985). The amino terminal of the A_1 chain in intact cholera toxin is hidden from monoclonal antibodies against the A_1 chain (Kurosky *et al.*, 1982a). Only after dissociation of the A subunit with thiol and SDS (such as is used to release the enzymic activity of the toxin) could antibodies be raised against the amino terminus (Kurosky *et al.*, 1982b). Activation therefore appears to make the amino terminus more accessible. This is consistent with the idea that the amino terminal residues, while they may not constitute the whole of the A_1 chain's active site, may form a part of the catalytic site or at least the binding site for NAD^+. In this way photoexcitation of the NAD^+ may have caused its incorporation into one of the four amino-terminal residues. More precise sequence information of the radiolabelled peptide purified here may answer this question more fully.

6.3.5 Photoaffinity labelling as a tool for locating the active site

Photoaffinity labelling is shown here to be a useful tool in locating the active site of an enzyme. When diphtheria toxin was labelled in a similar manner, the NAD^+ became covalently linked to residue 148, a glutamate within the active A_1 fragment (Carroll and Collier, 1984; Carroll *et al.*, 1985). Subsequent analysis of site-directed mutants showed that when this glutamate was replaced by an aspartate (a residue of similar size and charge which was unlikely

to have altered the protein's conformation), catalytic activity was abolished. Photoaffinity labelling had accurately identified a residue within the toxin's active site which was crucial for catalytic activity. This has implications for the suitable design of vaccines, which could be prepared from biologically inactive, but immunogenic forms of bacterial toxins such as cholera toxin. (The currently used vaccine for cholera toxin is made from killed whole organisms and is only 30% effective for 3 - 6 months).

However, it has not been proved here that the photolabelled preparation of toxin lacks activity, which would be expected if the active site were involved. While such a loss of activity would not prove unequivocally that the active site was labelled (the modification could have altered the protein's conformation for instance), it would strongly suggest that this was the case. The saturable nature of the incorporation and the specificity (only one peptide was labelled) suggest that the NAD^+ has become incorporated into either the binding site or the active site itself. However, care should be taken in interpreting results from this kind of experiment. In general, photoaffinity labelling tends to be more efficient when the ligand binds tightly to the protein, with non-specific binding more of a problem for ligands with K_d values above 100μM. But the rate of dissociation and the half-life of the photo-activated form of the ligand are also important. If the half-life is long ($>10^{-5}$ sec) and the rate of dissociation is fast then the photogenerated species may have time to diffuse some distance from its site of photoactivation and to label distant residues. Even for tightly bound complexes such as carbonic anhydrase and the

photo-affinity label p-azidobenzene sulphonimide (K_i = 10^{-6}M) photolysis leading to covalent labelling reportedly ocurred at sites some distance from the active site (Hixson and Hixson, 1975). The indiscriminate nature of the photolabelling process itself might be expected to produce a range of chemically similar species. Photolabelling of cholera toxin's A_1 chain has been reported before (Wisnieski *et al.*, 1979; Wisnieski and Bramhall, 1981). But in this case the photoreactive probe was not directed towards the active site. The probe 12-(4-azido-2-nitrophenoxy) stearoylglucosamine [1^{14}C] was used instead to show that the A_1 chain of the toxin could insert into membrane bilayers, where it became photolabelled. The actual residue which had become labelled was not so important.

In conclusion then, while photoaffinity labelling has led to the purification of a tryptic peptide of cholera toxin's A_1 chain containing a covalently-linked NAD^+ residue, care should be taken not to over-interpret the results of what can be a fairly indiscriminate technique. It is also worth noting that the photolabelling procedure itself may lead to anomalous results when the labelled protein is analysed using conventional methods of separation and analysis.

6.4 Conclusions and future work

The overwhelming impression gained from these studies must be that cholera toxin has a strikingly high K_m for its substrate NAD^+, a point reflected also in the K_d calculated here to be of the order of 4mM. There are no other reports of ADP-ribosyl transferases with K_m values of this order; diphtheria toxin, exotoxin A, pertussis toxin, turkey erythrocyte transferase; they all have K_m (and K_d values)

between 5 and 25μM. There is little indication as to why the K_m for NAD^+ should be quite so high in the case of cholera toxin. Findings outlined in Chapters Three and Five (the variable nature of K_m values for both substrates and the ability of both NAD^+ and an analogue of the second substrate (N_a) to bind to the toxin in the absence of the other substrate) are consistent with a random order rapid equilibrium kinetic mechanism proposed for cholera toxin by Osborne *et al.*, (1985). Certainly the phenomonen whereby the presence of one substrate decreases the toxin's affinity for the other substrate is an unusual one and has not been reported for other bacterial toxins. This unusual mechanism might seem at first to have some bearing on the high K_m value for NAD^+. However, an identical mechanism was proposed by the same authors for an avian ADP-ribosyl transferase (arginine-specific like cholera toxin) which had an estimated K_m of 8μM for NAD^+. The mechanism of action itself cannot be the sole cause of this high K_m value. In view of the likely cellular concentration of NAD^+ (around 70μM) an immediate thought is that NAD^+ itself cannot be the *in vivo* substrate, but rather a closely related derivative of NAD^+ may be the preferred substrate. However, analysis of a number of derivatives and analogues of NAD^+, both as replacements for NAD^+ in binding and kinetic experiments showed NAD^+ itself to be the best alternative. Unless there is some unidentified derivative of NAD^+ within intestinal epithelial cells, this proposal does not seem likely. Perhaps the answer lies in the site of action of the toxin's A_1 chain. There is a growing body of evidence that the A_1 chain may not only penetrate the membrane to allow entry into the cell but may

catalyse its ADP-ribosylation of N_s either close to or partially inserted into the plasma membrane. Van Heyningen, in demonstrating the activation of adenylate cyclase (van Heyningen, 1977) by covalently cross-linked toxin, provided evidence suggesting that the A and B subunits of the toxin may not have to become fully dissociated *in vivo*. Problems encountered in Chapter Three involving the activity of isolated A_1 (or rather the lack of it) did suggest that when the A_1 chain is kept on its own, it loses activity perhaps as a consequence of its complete dissociation from the other portions of the molecule. One theory might be that the A_1 chain is pushed through the membrane by some action of the rest of the toxin elicited by binding to G_{M1} on the cell's surface (for instance a large conformational change). It may penetrate the inner surface of the membrane just far enough to catalyse the ADP-ribosylation of N_s with the help of the numerous cofactors so far identified. What bearing this kind of mechanism might have on the availability of NAD^+ is not clear. Certainly, reports that some membrane proteins require a lipid environment for full activity (for instance N_s itself) imply that the hydrophobic nature of the membrane may promote the formation of certain protein conformations or stabilise others.

Exposure of mixtures of NAD^+ and cholera toxin to ultraviolet light resulted in Chapter Six in the covalent incorporation of the NAD^+ into a specific portion of the toxin's A_1 chain. This portion has been tentatively identified to be at the amino terminus of the A_1 chain, a portion which shows remarkably strong homology to the related *E.coli* heat labile toxin. As the phenomonen of photoinduced

incorporation has been reported for other toxins (diphtheria toxin and exotoxin A), the technique may provide a way of comparing the architecture of these enzyme's active sites. For instance the active site of *E.coli* LT seems likely to be similar, if not identical to that of cholera toxin as they are homologous and catalyse the same reaction. But do cholera toxin and LT resemble diphtheria toxin and exotoxin A? Functional domains responsible for a particular property, such as substrate binding may be shared between proteins, e.g. the Rossman fold domain of dehydrogenases. Both diphtheria and cholera toxins have intrinsic NAD'ase activity (the hydrolysis of NAD^+). This suggests that they can bind NAD^+ in such a way that its nicotinamide-ribose bond is exposed to nucleophilic attack. This particular orientation of binding may be enough to promote photoactivation of the NAD^+ in a certain way on exposure to ultraviolet light of a particular wavelength. Similar photoincorporation may well turn out to be a property of other ADP-ribosyl transferases, both bacterial and eukaryotic.

Work for the future might be found in defining exactly the site of photoincorporation of NAD^+ into cholera toxin and in using the technique to compare the active sites of different transferases. The role of cellular cofactors in the *in vivo* action of cholera toxin provides much scope for study as does the entry process for the A_1 chain. Also, the use of cholera toxin and pertussis toxin as tools in the study of G-proteins and their role in cellular-transduction processes seems likely to prove invaluable in defining more exactly the role of these processes in cellular signalling and perhaps even in the events surrounding oncogenic transformation.

APPENDIX A ANALYSIS OF RESULTS

a. Binding results

In equilibrium dialysis it is assumed that after equilibrium is established then the activity (taken to be equal to concentration in this case) of free ligand is equal on both sides of the membrane. By measuring the concentration of ligand on both sides of the membrane b (bound ligand in mol l^{-1}) and f (free ligand in mol l^{-1}) can be calculated. The binding parameters of K_d and n can then be calculated using a computer program based on the non-linear expression of b and f using Marquardt's aligorithm (Marquardt, 1963) or from the linear regression of 1/r on 1/f.

b. Kinetic data

Initial velocities for the enzyme catalysed release of nicotinamide from NAD^+ were calculated from linear progress curves of the reaction rate (Cornish-Bowden, 1975). Michaelis constants, maximum velocities and inhibition data were calculated using the non-parametric method of Cornish-Bowden and Eisenthal (1978) fitted by the method of Marquardt (1963).

Interactive programs were written in IMP and run on the ICL 2988 at the Edinburgh Regional Computing Centre.

APPENDIX B ABBREVIATIONS

A	- adenine
A_1	- the A_1 chain of cholera toxin
Ap^r	- ampicillin resistance
ACTH	- adrenal corticotrophic hormone
ADPR, ADP-ribose	- adenosine diphosphoribose
APAD	- acetyl pyridine adenine dinucleotide
A599	- [12-(phenylmethylamino) dodecanyl] guanidine
BSA	- bovine serum albumin
CT	- cholera toxin
DNS	- dansyl
DTT	- dithiothreitol
EF-2	- elongation factor 2
G_{M1}	- galactosyl-N-acetyl galactosaminyl-[N-acetylneuraminyl]-galactosylglucosyl-ceramide
Gpp(NH)p	- 5^1-guanylyl-imidodiphosphate
hplc	- high pressure liquid chromatography
LT	- *E.coli* heat labile toxin
NAD'ase	- NAD^+ glycohydrolase
Nbf-Cl	- 4-chloro-7-nitrobenzofuran
N_i	- the inhibitory regulatory subunit of adenylate cyclase
$N_{i\alpha}$	- the α subunit of N_i
N_s	- the stimulatory regulatory subunit of adenylate cyclase
$N_{s\alpha}$	- the α subunit of N_s
N_β	- the β subunit of N_s and/or N_i
PITC	- phenylisothiocyanate
PMSF	- phenylmethylsulphonyl fluoride

PTC	- phenylthiocarbamyl
ROS	- rod outer segments of the retina
SDS	- sodium dodecyl sulphate
12% SDS-PAGE	- 12% polyacrylamide gel electrophoresis in the presence of SDS
TEMED	- N, N, N^1, N^1-tetra methylethylene diamine
tlc	- thin layer chromatography
thio-NAD^+	- thionicotinamide adenine dinucleotide
4406	- [10-(phenylmethylamino) decanyl] guanidine

APPENDIX C THE PRIMARY STRUCTURE OF CHOLERA TOXIN A SUBUNIT

The disulphide bridge is shown and the point of cleavage between A_1 and A_2 chains.

ASN-ASP-ASP-LYS-LEU-TYR-ARG-ALA-ASP-SER-ARG-PRO-PRO-ASP-GLU-ILE-LYS-GLN-SER-GLY- 20

GLY-LEU-MET-PRO-ARG-GLY-GLN-SER-GLU-TYR-PHE-ASP-ARG-GLY-THR-GLN-MET-ASN-ILE-ASN- 40

LEU-TYR-ASP-HIS-ALA-ARG-GLY-THR-GLN-THR-GLY-PHE-VAL-ARG-HIS-ASP-ASP-GLY-TYR-VAL- 60

SER-THR-SER-ILE-SER-LEU-ARG-SER-ALA-HIS-LEU-VAL-GLY-GLU-THR-ILE-LEU-SER-GLY-HIS- 80

SER-THR-TYR-TYR-ILE-TYR-VAL-ILE-ALA-THR-ALA-PRO-ASN-MET-PHE-ASN-VAL-ASN-ASP-VAL- 100

LEU-GLY-ALA-TYR-SER-PRO-HIS-PRO-ASP-GLU-GLN-GLU-VAL-SER-ALA-LEU-GLY-GLY-ILE-PRO- 120

TYR-SER-GLN-ILE-TYR-GLY-TRP-TYR-ARG-VAL-HIS-PHE-GLY-VAL-LEU-ASP-GLU-GLN-LEU-HIS- 140

ARG-ASN-ARG-GLY-TYR-ARG-ASP-ARG-TYR-TYR-SER-ASN-LEU-ASP-ILE-ALA-PRO-ALA-ALA-ASP- 160

GLY-TYR-GLY-LEU-ALA-GLY-PHE-PRO-PRO-GLU-HIS-ARG-ALA-TRP-ARG-GLU-GLU-PRO-TRP-ILE- 180

HIS-HIS-ALA-PRO-PRO-GLY-CYS-GLY-ASP-ALA-PRO-ARG | SER-SER | MET-SER-ASN-THR-CYS-ASP- 200

GLU-LYS-THR-GLN-SER-LEU-GLY-VAL-LYS-PHE-LEU-ASP-GLU-TYR-GLN-SER-LYS-VAL-LYS-ARG- 220

GLN-ILE-PHE-SER-GLY-TYR-GLN-SER-ASP-ILE-ASP-THR-HIS-ASN-ARG-ILE-LYS-ASP-GLU-LEU 240

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